GENETIC REGULATION OF ANGIOGENESIS AND LYMPHANGIOGENESIS

Visualization and characterization of the developing embryonic vasculature

The research in this thesis was performed at the Hubrecht Institute of the Royal Netherlands of Arts and Sciences (KNAW), within the framework of the graduate school of Cancer Genomics and Developmental Biology in Utrecht. Most of the research was performed in close collaboration with the department of Experimental Cardiology of the Erasmus University Medical Centre, within the framework of the COEUR research graduate school in Rotterdam, The Netherlands.

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Contact: frank.l.bos@gmail.com

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DE GENETISCHE REGULATIE VAN ANGIOGENESE EN LYMPHANGIOGENESE

Visualisatie en karakterisatie van het ontwikkelende embryonale vatenstelsel

Proefschrift

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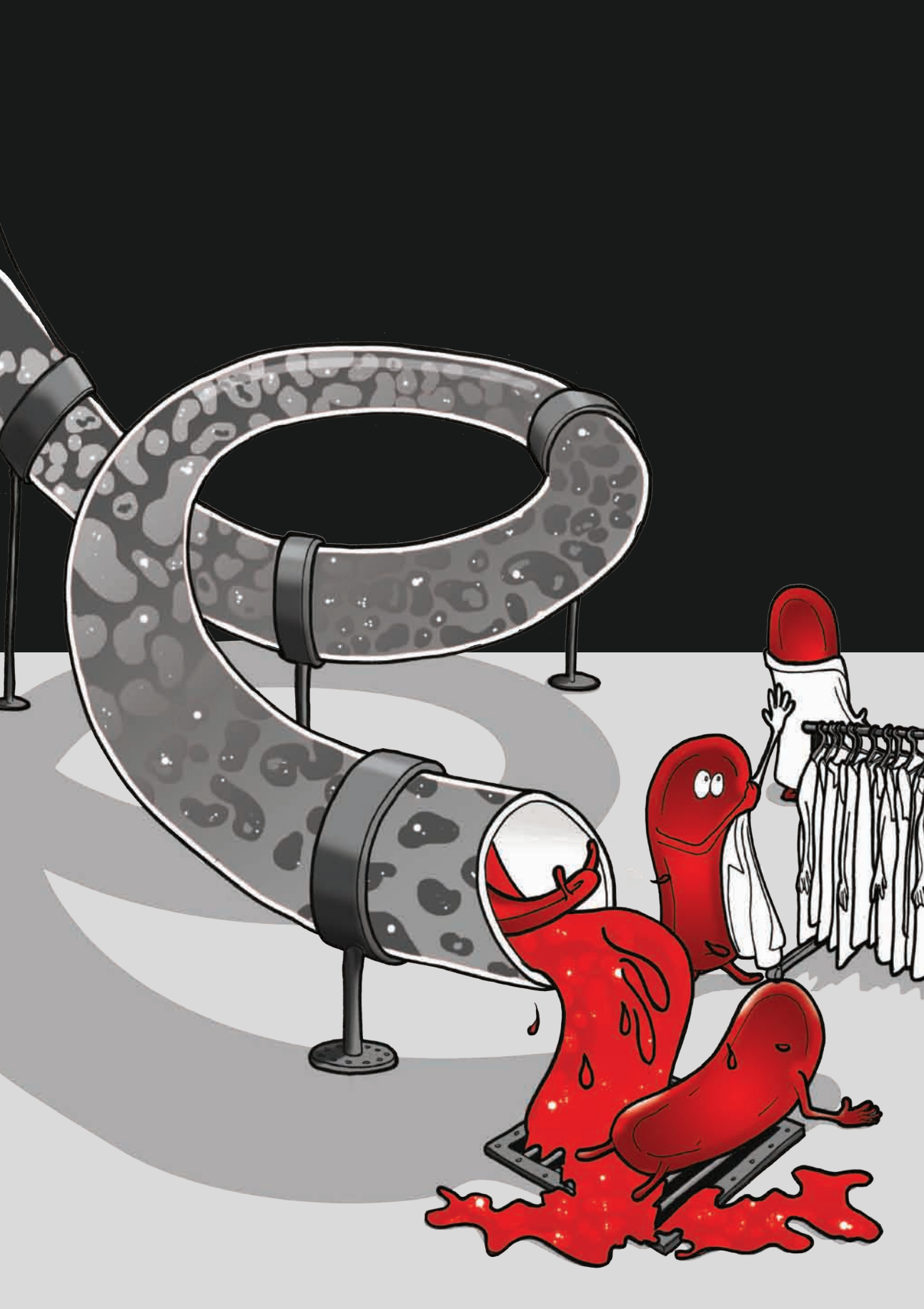
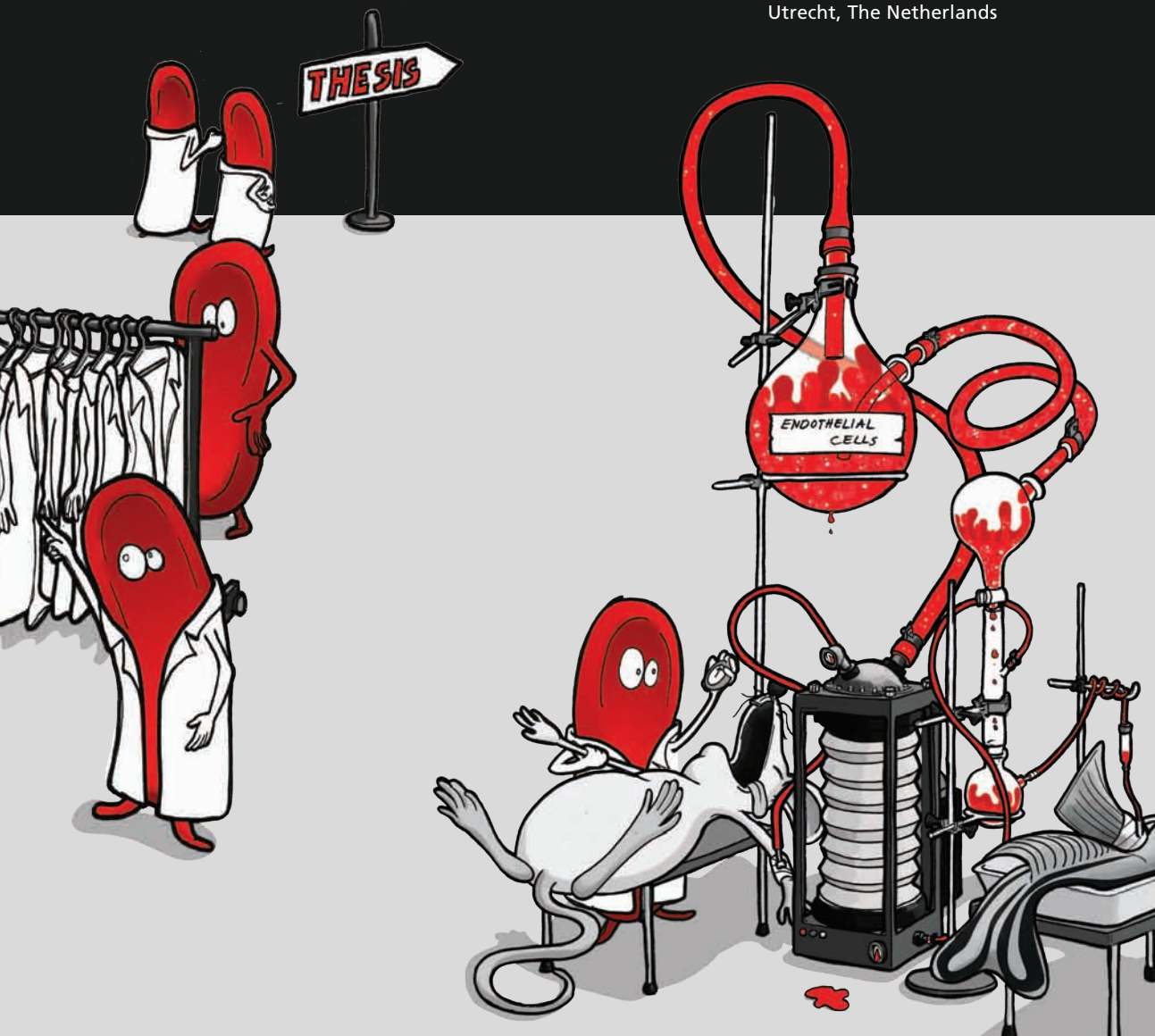


1

INTRODUCTION TO VASCULOGENESIS, ANGIOGENESIS AND LYMPHANGIOGENESIS

Frank L. Bos[1,2], Stefan Schulte-Merker[2] & Henricus J. Duckers[1,2]

1. Experimental Cardiology, Thoraxcenter, Erasmus University Medical Center, 's-Gravendijkwal 230, 3015 CE Rotterdam, The Netherlands
2. Hubrecht Institute-KNAW & University Medical Centre, Utrecht, Uppsalalaan 8 3584 CT, Utrecht, The Netherlands



The circulatory system

Aelius Galenus (AD 129 – 199) was one of the first persons to explore the blood vasculature. During his work as physician and surgeon, he recognized distinct differences in blood vessels. During surgery, he observed that vessels were filled with either dark or bright blood. He believed that the human blood vascular system contained two one-way blood distribution routes. The dark (venous) blood was generated directly from food uptake in the liver, whereas the bright (arterial) blood was generated in the heart. From the heart and liver, blood was then equally distributed and 'consumed' by all other organs in the body. To complete the vascular system, blood was then re-generated in either the heart or liver (reviewed in (Carmeliet, 2005).

This 'two-one way circulation' theory was believed for centuries and it was only until the 16th century that the British biologist and medical doctor *William Harvey* could show that *Galenus* was wrong. *Harvey* characterized and quantified the blood volume which passes the heart and concluded that this was much larger than the amount of blood that could be generated by the body itself (On the Motion of the Heart and Blood in Animals, 1628, William Harvey). *Harvey* postulated that there had to be a circulatory loop in the body which consisted of the heart and a connected vessel system. With a simple experiment, by tightening a ligature on to the upper arm of an individual, he indeed identified a circulatory loop which was connected to the heart and identified the presence of arteries and veins but also functional differences between arteries and veins (On The Motion Of The Heart And Blood In Animals, 1628, William Harvey, (Carmeliet, 2005).

Nowadays, we know that the human circulatory system consists of the heart, blood vessels and the lymphatic system. The circulatory system is subdivided into the cardiovascular system and the lymphatic system (even though it is sometimes argued that the lymphatic system is not part of the circulatory system, since it is not a closed circulatory loop). The cardiovascular system consists of the heart and blood vessels that propel and conduct blood through the body (Clinically Orientated Anatomy, 4th Edition). In parallel, the lymphatic system provides a network that is able to withdraw excess tissue fluid from the interstitial space (Figure 1 A). The cardiovascular system provides oxygen and nutrients to all organs and tissues in the body. First, oxygen-rich blood leaves the heart under high pressure and is distributed to organs via the thick-walled arteries. These arteries branch into arterioles and capillaries that make up the capillary bed. Here, the blood pressure is lower and exchange of gases, hormones and nutrients can occur. After the exchange, blood cells enter the thin veins of the venous system that eventually drain to larger veins and back to the heart. The heart first pumps the blood through the lungs where it can be re-oxygenized before it enters the heart again (Figure 1 A). Due to high blood pressure in arteries, fluid can leak out of vessels and will then accumulate in the interstitial space. About 90% of this fluid is taken up again by veins, whereas the remaining 10% is taken up by lymphatic capillaries, small blunt ended vessels, which constitute part of the lymphatic system (Alitalo et al., 2005; Tammela and Alitalo, 2010). These small lymphatic capillaries

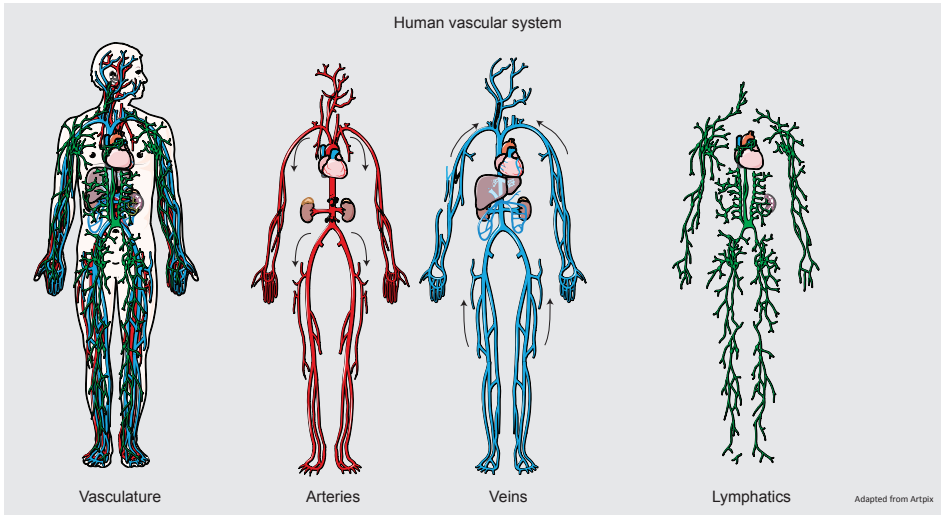


Figure 1: The human circulatory system. The human circulatory system consists of the heart, blood vessels (arteries and veins) and the lymphatic system. It is subdivided in a cardiovascular system (red/blue) and a lymphatic system (green). The cardiovascular system consists of the heart and blood vessels.

drain towards the bigger lymphatics such as the thoracic duct where lymph is filtered in the lymph nodes (Tammela and Alitalo, 2010). In addition to the drainage function of the lymphatic system, it also functions as the primary trafficking route of immune cells. Furthermore, lymphatics are important for the absorption and transport of dietary lipids. The lymphatic system therefore also consists of non-vessel structures, such as circulating lymphocytes, spleen and lymph nodes (Karpanen and Alitalo, 2008; Makinen and Alitalo, 2007).

Endothelial cells build the vascular tree

All embryonic and mature vascular structures consist of a thin layer of endothelial cells (EC). ECs are derived from hemangioblasts (endothelial and haematopoietic precursors) that are not fully differentiated and still retain some endothelial as well as hematopoietic cell properties (Fong et al., 1999). The differentiation of hemangioblasts to endothelial cells is, among many others, under control of the vascular endothelial growth factor A (VEGF-A). VEGF-A is part of a large family of vascular and angiogenic regulators such as VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor (PlGF) (Figure 2A, B) (Ferrara et al., 2003).

The development of the first cord-like structures starts with the assembly of angioblasts in a process called *vasculogenesis,* which is *de novo* synthesis of blood vessels and requires VEGF-A. The balance between different VEGF-A isoforms and their receptors is further required for proper development of these first blood vessels. For example, VEGF-A is able to bind to the extracellular domain of the vascular

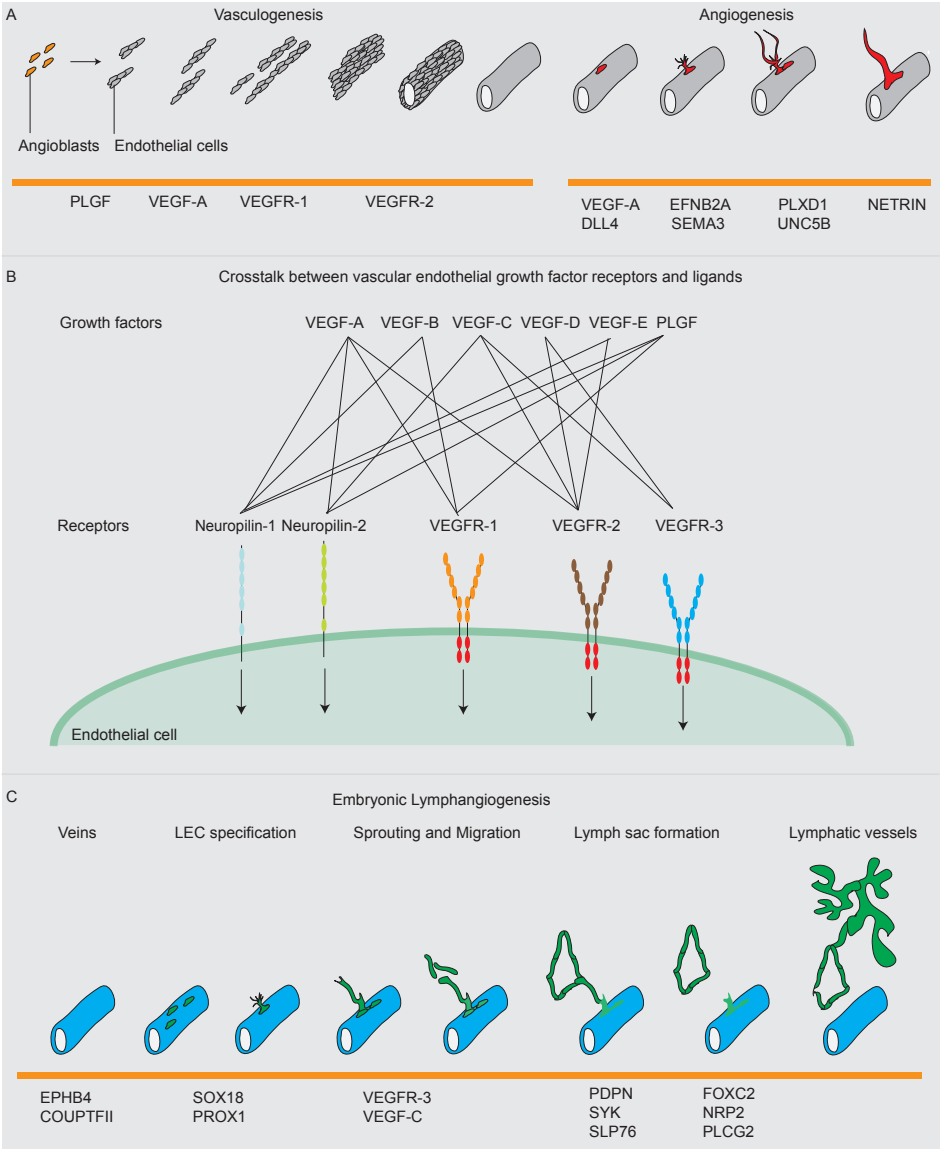


Figure 2: Genetic regulation of vascular structures. A. Development of the first cord-like structures starts with the assembly of angioblasts in the embryonic midline (*vasculogenesis),* which is *de novo* generation of blood vessels and requires VEGF-A, PlGF and the VEGF-receptors VEGFR-1 and VEGFR-2. Angiogenesis is defined as the process of generating new vessels from already existing ones (often through sprouting and migration). B. Cross talk between different VEGF ligands and receptors which regulate vasculogenesis and angiogenesis. C Genetic regulation of the key steps of lymphangiogenesis: the specification, budding, sprouting and migration of lymphatic endothelial cells in the process of lymph sacs formation.

endothelial growth factor receptor 2 (VEGFR-2), thereby activating downstream effectors that promote the differentiation, sprouting and further proliferation of ECs. VEGF-A is also capable of binding VEGFR-1 with relatively high affinity. However, the kinase activity of VEGFR-1 is much weaker than that of VEGFR-2 (Yamashita et al., 2000). Thus, by competing for the binding of the available VEGF, VEGFR-1 is a counter player of VEGFR-2 (Shibuya, 2006a; Shibuya, 2006b). Both receptors are important for the development of the vasculature, since the knock-out mice for VEGFR-1 and VEGFR-2 are both embryonic lethal due to vascular defects (Fong et al., 1999; Shalaby et al., 1997; Takahashi and Shibuya, 2005). In general, there is a lot of crosstalk between various VEGF ligands and the VEGF receptors, all required for proper vascular development (Figure 2B) (Takahashi and Shibuya, 2005). After the establishment of the very first embryonic vessels, endothelial cells sprout and migrate from the existing blood vessels, a process referred to as angiogenesis. Vasculogenesis, angiogenesis and extensive remodeling will eventually give rise to the complete vasculature with fully differentiated arteries and veins. Arteries are covered with a thick layer of smooth muscle cells which are able to contract and react and compensate for the hemodynamic changes. The venous system returns blood to the heart, the blood pressure is much lower and does not require a thick layer of smooth muscle cells. In addition, veins contain valves to prevent backflow of blood (Adams and Alitalo, 2007).

Arteries and Veins

Although arteries and veins consist of ECs, their gene expression profile is different. The specification of an EC is regulated by many developmental programs. ECs can acquire an arterial or a venous fate and one of the master switches of EC fate is the Notch pathway. Notch signaling is required for proper vascular morphogenesis and drives arterial fate determination (Ishiko et al., 2005; Krebs et al., 2004; Krebs et al., 2000). VEGF-A is also essential for arterial EC differentiation. Upon VEGF-A activation, the ECs express higher levels of arterial markers such as the Notch delta like ligand 4 (DLL4) and the transmembrane protein Ephrin B2 (EFNB2). Ephrin B2 can bind multiple receptor tyrosine kinases such as the receptor EphB4, which is solely a venous endothelial marker. Therefore, EphrinB2 and EphB4 are markers for arterial and venous identity of ECs respectively, and they are also functionally important for angiogenesis (Figure 2A) (Himanen and Nikolov, 2003).

Next to VEGF-A, EphrinB2 and DLL4, members of the Neuropilin (Nrp) family (of proteins) are also involved in arterio-venous specification. Neuropilin-1 (NRP1) is restricted to arterial endothelial cells (Mukouyama et al., 2005), whereas Neuropilin-2 (NRP2) is restricted to venous endothelium (Yuan et al., 2002). Since both Neuropilin receptors can be bound to VEGF ligands, they are both modulators of the cellular response to members of the VEGF stimuli (Figure 2B).

Furthermore, the specification of veins is dependent on CoupTFII (Nr2f2), a member of the orphan nuclear receptor superfamily. CoupTFII is expressed in venous

endothelium and mice lacking this receptor acquire an arterial fate in endothelial cells by upregulating the arterial markers DLL4 and EphrinB2.

Establishment of the lymphatic vasculature

In mice, after the establishment of the primary blood vasculature, the lymphatics are established. The lymphatic vasculature consists of lymphatic endothelial cells (LECs) (Figure 2C), which are distinct in their transcriptional characteristics from other ECs (You et al., 2005). More than a century ago, the medical doctor Florence Sabin (1902) proposed that the lymphatic vasculature was derived from venous endothelial cells. It was only until 2007 that this finding was confirmed with lineage tracing and mutant mice experiments (Srinivasan et al., 2007). As noted earlier, the orphan receptor CoupTFII is a master regulator of venous EC fate. Hence, loss of CoupTFII disrupts overall venous identity of ECs and therefore all ECs switch to the arterial cell fate which, as a secondary consequence, results in a dramatic loss in LECs (Srinivasan et al., 2007). Similar to BECs, LECs are able to respond to specific inducing extracellular signals to acquire a LEC fate, and VEGF-C is the most prominent factor in that respect.

At E9.75, a subpopulation of venous cells starts to express lymphatic vessel endothelial hyaluronan receptor (Lyve-1)(Banerji et al., 1999; Luong et al., 2009), and prospero homeobox gene Prox1 (Hong and Detmar, 2003; Hong et al., 2002; Wigle et al., 2002; Wigle and Oliver, 1999).The transcription factor Prox1 is necessary and sufficient to drive LEC specification (Hong et al., 2002; Wigle et al., 2002). Prox1 expression in venous ECs is determined by transcriptional activators such as CoupTFII (Oliver and Srinivasan, 2010; Srinivasan et al., 2007) and SRY (sex determining region Y)-box 18 (Sox18) (Downes et al., 2009; Francois et al., 2008).Taken together, CoupTFII, Sox18 and Prox1 are essential for LEC differentiation in mammals. Lack of either one of these genes results in a complete lack of LECs and therefore the lymphatic vasculature is not established (Figure 2 C).

Sox18 is found to be mutated in human patients who suffer from *hypothichosis-lymphedema-telangiectesia*, which is characterized by swelling of extremities (lymphedema). Sox18 targeted mice suffer from various phenotypes, depending on the genetic background. Strikingly, Sox18 expression was initially found in arterial endothelial cells, but has recently been discovered in a subset of endothelial cells in the cardinal vein around E9.0, and Sox18 acts as a direct activator of Prox1 in these cells (Downes et al., 2009; Francois et al., 2008). Endothelial cells in Sox18-depleted mice do not acquire LEC specification fates, due to the absence of Prox1 expression in venous endothelium. As a result, the lymphatic vasculature is not initiated and mice die *in uteri*. Sox18 (and therefore indirectly also Prox1) is, in turn, directly activated by CoupTFII. Hence, mice are also devoid of the lymphatic vasculature when CoupTFII is deleted, showing that CoupTFII is also crucial during the activation of Sox18 and Prox1 towards a LEC differentiation state (Oliver and Srinivasan, 2010).

Differentiated LECs migrate away from veins and respond to guidance cues, whereas venous EC do not respond and remain within the venous endothelium (Oliver

and Srinivasan, 2010). One of these cru guidance molecules and migration factors is the secreted protein VEGF-C (Figure 3). VEGF-C is expressed by mesenchymal cells and vascular smooth muscle cells adjacent to arteries and veins. The finding that Prox1-positive LECs fail to migrate in VEGF-C null mice, suggests that VEGF-C is not required for the specification of LECs, but can induce proliferation and migration of LECs (Caunt et al., 2008; Karkkainen et al., 2004). VEGF-C can bind to VEGFR-3 and is required for guidance of and migration of LECs. VEGFR-3 expression can be detected

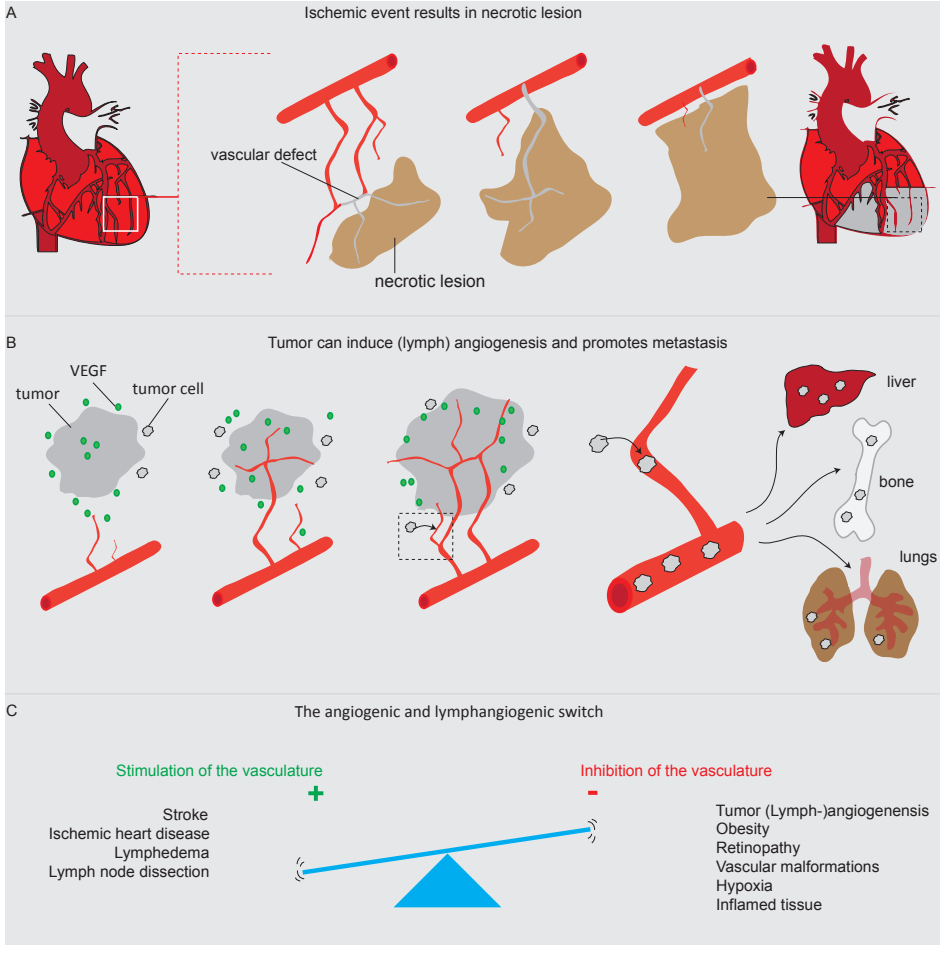


Figure 3: Vasculature associated diseases. (A). Atherosclerosis can result in the formation of plaques and cause thrombus formation, which can result in hypo-oxygenation of tissues and cause ischemic events. B. During cancer progression, tumors secrete growth factors, which can attract blood vessels and further accelerate the growth of tumors. Metastasizing cancer cells can subsequently spread easily towards different organs and tissues. C. The angiogenic switch of vascular associated diseases. Stimulation or inhibition of either blood or lymphatic vessels can improve clinical outcome of the vascular associated diseases.

in venous endothelium, but becomes restricted towards LECs during late stages of development and adulthood. Patients with VEGFR-3 mutations acquire lymphedema supporting the notion of the crucial role of this receptor in lymphangiogenesis (Connell et al., 2009; Futatani et al., 2008). Taken together, LEC fate is dependent of Prox1, but VEGFR-3 and VEGF-C are both required for the proper migration of these cells. In addition, VEGFR-3 knock-out mice show cardiovascular abnormalities during embryonic development, before the actual onset of the lymphatic vasculature (Dumont et al. 1998), indicating that VEGFR-3 plays multiple crucial roles in development.

Lymphangiogenesis is defined as the specification of venous ECs into LECs. Budding, sprouting and migration of LECs eventually results into the development of lymphatic sacs and, subsequently, the lymphatic vasculature. Until now, there are only a few genes discovered that regulate the early steps of this process, whereas numerous genes are known to be required for the maturation of lymphatics. Maturation defects result in subtle and non-lethal phenotypes which involve abnormal patterning, hypertrophy, reduced lymphatic number and abnormal connections (reviewed in (Adams and Alitalo, 2007). In this thesis we mainly focus on the primary embryonic lymphangiogenesis and therefore we will not further describe genes that affect maturation defects in detail and are reviewed elsewhere (Tammela and Alitalo, 2010).

Vascular disease

The circulatory system is often implicated in disease and, since the last decade, cardiovascular disease (CVD) is the major cause of death globally. CVD represents a group of diseases which affect the vasculature and the heart. Here we describe a few of them.

Arteriosclerosis is associated with thickening of vessel walls and loss of elastic strength, thereby causing abnormal functioning of vessels. Atherosclerosis is associated with the buildup of fat in the arterial walls, which can form plaques and cause thrombus formation (Figure 3A). A major consequence of arteriosclerosis is the loss of oxygen supply to vital organs which eventually results in an ischemic event. For example, ischemic heart disease is caused by insufficient blood supply to the heart and often results in myocardial infarction or stroke (Adams and Alitalo, 2007; Alitalo and Carmeliet, 2002).

Furthermore, the vasculature plays an important role in the progression of cancer (Folkman, 2002; Folkman and Ingber, 1992). During tumor growth, tumor cells require a dramatically increased supply of oxygen to divide and multiply. During cancer progression, tumors secrete growth factors such as VEGF-A to attract blood vessels. When new blood vessels are connected to the tumor, this will further accelerate tumor growth rates (Folkman, 1992). In addition, with the rapid growth and the connection to the circulatory system, metastasizing cancer cells can spread easily towards other organs. This process is referred to as metastatic spread (metastasis) and is a crucial part in cancer progression. Understanding the mechanisms of tumor angiogenesis has lead

to approval of anti-angiogenesis drugs for cancer treatment and other vascular diseases (Carmeliet and Jain, 2011; Folkman, 1971; Folkman, 2002).

Lymphedema

The lymphatic vasculature has also been implicated in a number of diseases. One of the characteristics of a dysfunctional lymphatic system is edema formation in body extremities. Lymphedema is classified in primary (congenital) lymphedema and secondary (or acquired) lymphedema. Although primary lymphedema is very rare, point mutations in the VEGFR-3 gene have been found and are the major cause of Milroy disease, which is characterized by abnormal lymphatic capillaries at birth (Connell et al., 2009). In addition, the transcription factor SOX18 is linked to *hypothichosis-lymphedema-telangiectasia* syndrome, characterized by rupturing of the blood vasculature and malformations in lymphatics. (Irrthum et al., 2003). This disease and phenotype was further studied in detail in the *Ragged* mutant mice, which also harbor a mutation in SOX18 (Downes et al., 2009). Furthermore, *lymphedema-distichiasis* is characterized by late onset edema and enlarged veins and is linked to the transcription factor FOXC2 (Maby-El Hajjami and Petrova, 2008; Mellor et al., 2007). The underlying mechanism of this disease was studied in mutant *Foxc2* mice. In these mice, lymphatic valves are missing and lymphatics have ectopic coverage of smooth muscle cells and basement membrane (Norrmen et al., 2009).

The majority of lymphedema cases are secondary and due to damaged lymphatic vessels (Nakamura and Rockson, 2008; Radhakrishnan and Rockson, 2008; Tammela and Alitalo, 2010). The most common example of lymphedema is *filariasis,* also known is *elephantiasis*. This disease is caused by a parasitic worm infection of lymphatics and affects approximately 100 million people worldwide. The infection leads to the blockage and scarring of lymphatic vessels which results in chronic lymphedema of lower limbs and genital organs. This infection triggers the immune response and stimulates the overproduction of lymphangiogenic factors such as VEGF-C, VEGF-D and VEGF-A, causing hyperplasia of lymphatics which result in non-functional lymphatics (Pfarr et al., 2009).

In cancer, tumor dissemination can occur towards the lymph nodes via the lymphatics, similar to the dissemination of cancer cells that extravasate via the blood stream to other organs (Figure 3B) (Stacker et al., 2001). With respect to those diseases implicated with the blood vascular or lymphatic system, it is crucial to understand how blood- or lymphatic vessels function and develop. Restoring functional vessels by stimulation of blood- and lymphatic vessel growth is required to treat several pathologies such as cardiovascular diseases like stroke or lymphedema. On the contrary, therapeutic intervention requires the complete inhibition of blood and lymphatic vessel supply (e.g. tumor formation). In both scenarios it is important to identify the regulators of blood and lymphatic vessel formation and discover therapeutic targets to provide therapeutic benefit for patients suffering from these diseases (Achen et al., 2006; Achen and Stacker, 2006; Stacker et al., 2006). Therefore, in this thesis, we aim to

gain insight into the genetic programs that are crucial and essential for the embryonic development of the (first) blood and lymphatic vessels to improve clinical outcome (Figure 3 B, C).

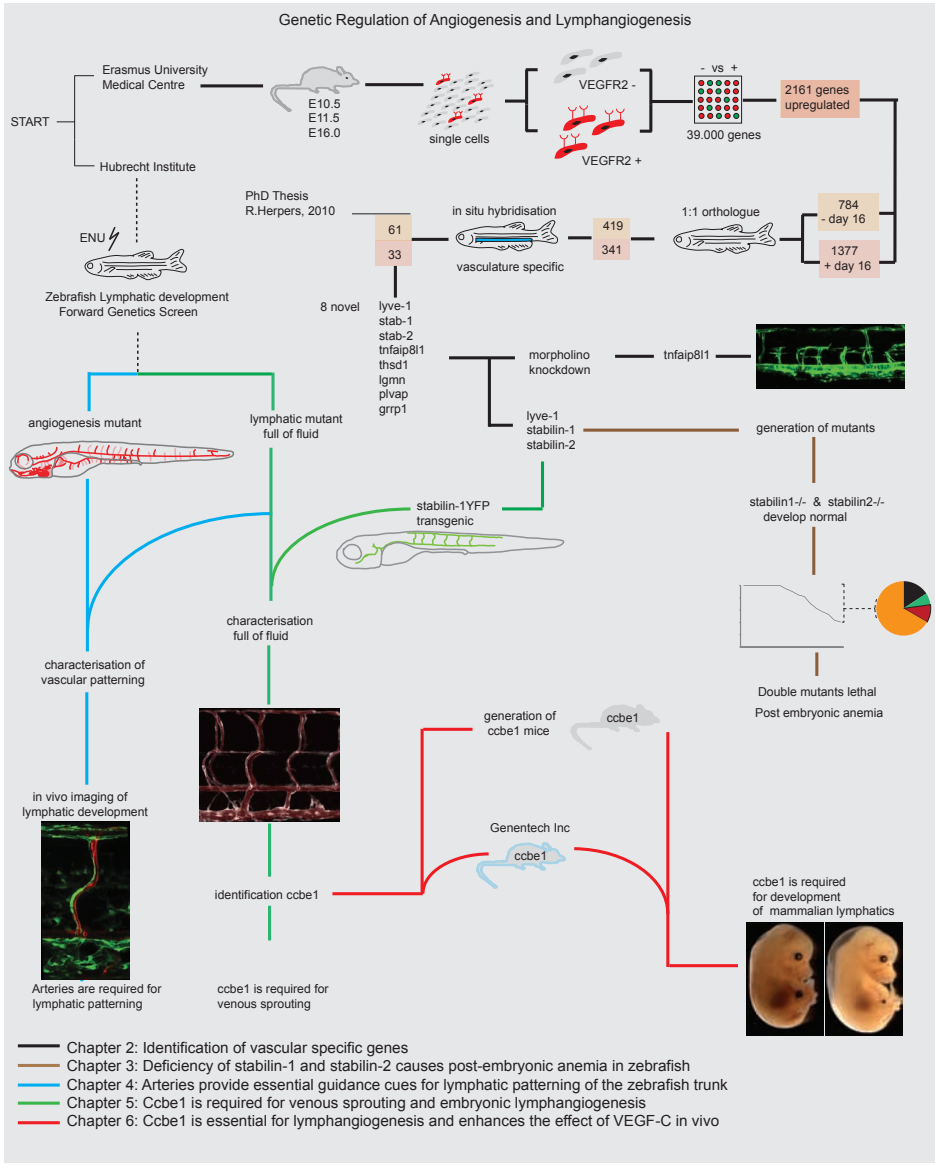
OUTLINE OF THIS THESIS

In **chapter 2**, we introduce a trans-species approach and make use of reverse genetics to identify novel regulators of vascular development. We isolated endothelial cells during crucial time points of murine vascular development and compared their genetic profiles with non-endothelial cells. Next, a large-scale *in situ* hybridization screen was performed in zebrafish and genes were selected based on expression patterns. A subset of endothelial expressed genes was subsequently targeted in an *in vivo* knockdown screen in zebrafish. In this chapter we describe zebrafish vasculogenesis and angiogenesis and report the identification and function of several zebrafish genes with endothelial expression.

In **chapter 3**, we report the characterization of the zebrafish vascular specific genes *stabilin-1* and *stabilin-2.* Since specific markers for zebrafish lymphatic development were lacking, we used the promoter of *stabilin-1* to generate a venous and lymphatic specific endothelial transgenic line: *stabilin-1:YFP.* During embryonic development, *stabilin-1* is initially expressed in most endothelial cells but becomes progressively restricted to venous and lymphatic endothelium and finally labels lymphatic structures until adulthood. In addition, we have generated mutants of *stabilin-1* and *stabilin-2* to address the function of *stabilin* genes in development. *Stabilin-1* and *stabilin-2* mutants are embryonic viable and show no vascular or lymphatic abnormalities. However, double mutants become anemic due to severely reduced numbers erythrocytes at 5 days post fertilization (dpf) and are embryonic lethal beyond 5 dpf. These results suggest that *stabilin* genes are not required for embryonic angiogenesis and lymphangiogenesis, but at least the presence of one of the stabilin genes is required for proper erythropoiesis.

In **chapter 4**, we set out to unravel the vascular and lymphatic patterning during zebrafish development. Here, we first report the generation and characterization of novel arterial and lymphatic transgenic lines. We used the *flt1* (VEGFR-1) regulatory elements to generate a line which specifically labels arterial endothelial cells. With this line, we created an atlas of the trunk vasculature and quantified the distribution of arteries and veins in zebrafish development. In addition, we used the lymphatic transgenic (*SAGG27FFC;UAS:GFP)* line to characterize the distribution of lymphatic vessels in the zebrafish trunk. With high resolution time lapse imaging we observed that all migrating lymphatic endothelial cells s aligned along arterial intersegmental vessels. Furthermore, we used mutant and morphants of the gene *kdrl* to show the requirement of arteries for proper lymphatic development in the zebrafish trunk.

In **chapter 5**, we report the identification and characterization of a novel zebrafish mutant from a forward genetic screen. The zebrafish mutant *full of fluid (fof)* lacks all aspects of the lymphatic vasculature. With the usage of the transgenic lines reported in chapter 3 *(stabilin-1:YFP*) and chapter 4 (*flt1^{enh}:RFP*), we were able to characterize

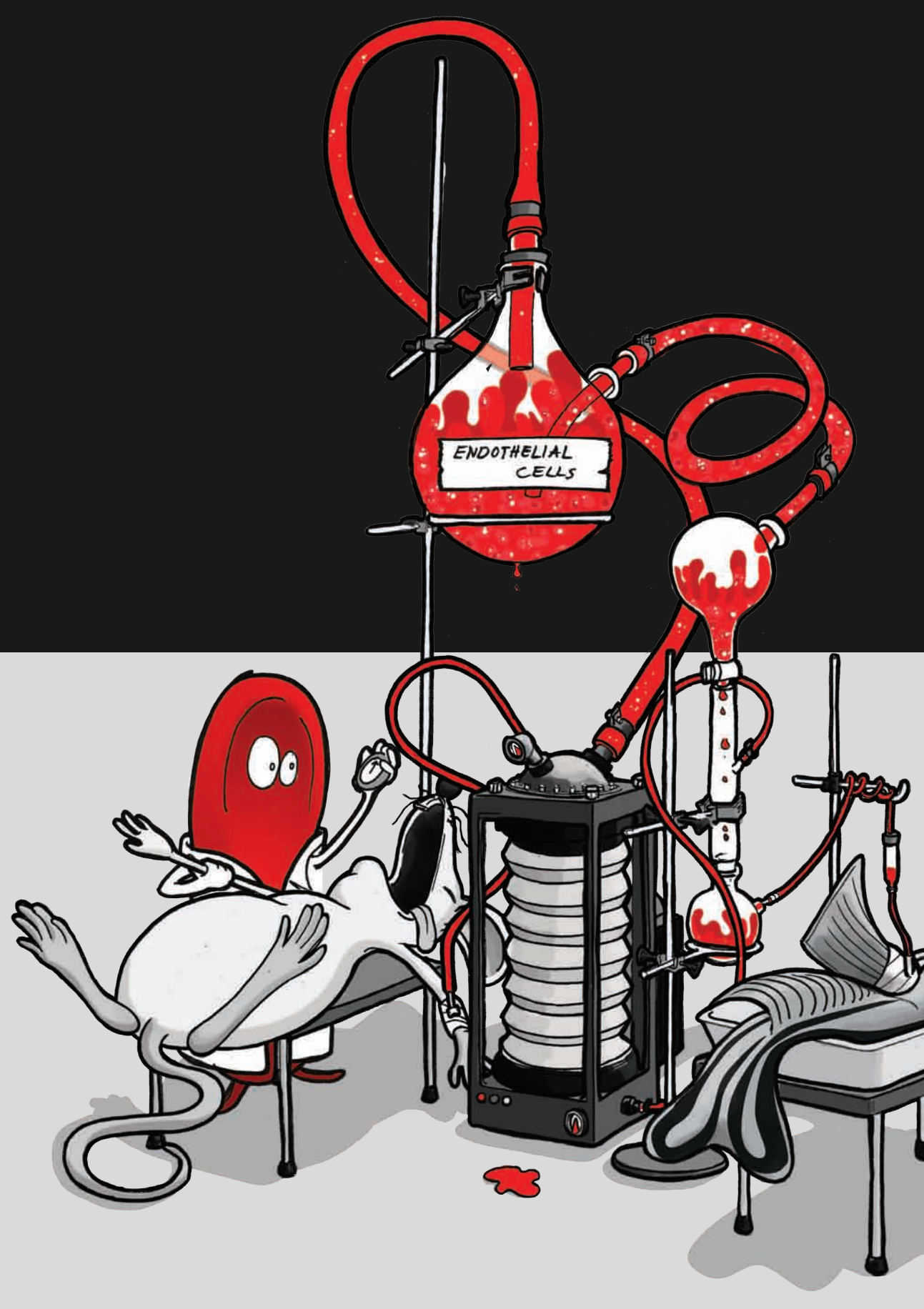


this mutant in detail. Mutants have a defect in venous angiogenic sprouting and lymphangioblast budding from venous endothelium. Furthermore, the responsible gene, *collagen and calcium binding epidermal growth factor domains-1 (ccbe1),* is predicted to encode a secreted protein. Ccbe1 is expressed along the migration routes of lymphatic endothelial cells and acts at the same developmental processes as *Vegfr-3/Vegf-c* signaling.

In **chapter 6**, we report the generation of two *Ccbe1* targeting strategies to characterize the mammalian function of *Ccbe1*. *Ccbe1*$^{-/-}$ mice have relatively normal

vascular and angiogenic patterning, but acquire severe edema and are embryonic lethal. *Ccbe1*$^{-/-}$ mice lack the complete lymphatic vasculature. In mutants, we observed that venous endothelial cells become specified as lymphatic endothelial cells (LECs), but fail to migrate away from embryonic veins. This migration acts independent of VEGFR-3 receptor activation, since the phosphorylation of the receptor appears unaltered in mutant LECs. In addition, we have generated recombinant human CCBE1 which moderately stimulates a lymphangiogenic response in the corneal micropocket assay, but above all enhances VEGF-C induced lymphangiogenic response.

In **chapter 7**, I conclude with a general discussion on zebrafish and mouse lymphangiogenesis and the potential role of CCBE1 as a target for either stimulation or inhibition of lymphangiogenesis to improve clinical outcome of patients suffering from lymphatic associated disease.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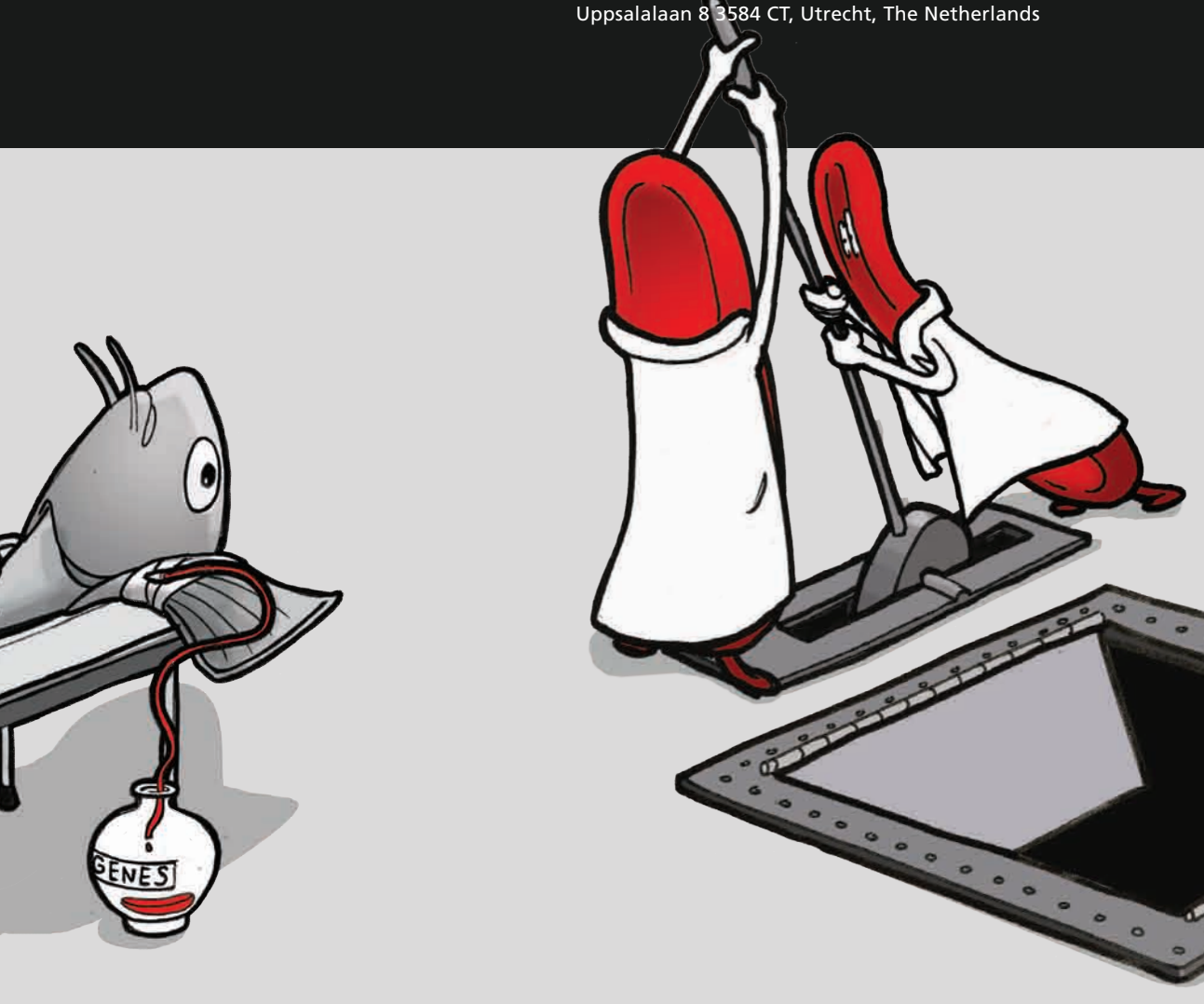
2

A CROSS-SPECIES APPROACH TO IDENTIFY NOVEL REGULATORS OF VASCULOGENESIS, ANGIOGENESIS AND LYMPHANGIOGENESIS

Frank L. Bos[1,2], Esther van de Kamp[1], Robert Herpers[1,2], Dennie Tempel[1,2], Caroline Cheng[1,2], Stefan Schulte-Merker[2] & Henricus J. Duckers[1]

1. Experimental Cardiology, Thorax center, Erasmus University Medical Center, 's-Gravendijkwal 230, 3015 CE Rotterdam, The Netherlands
2. Hubrecht Institute-KNAW & University Medical Centre, Utrecht, Uppsalalaan 8 3584 CT, Utrecht, The Netherlands



SUMMARY

Revascularization of ischemic tissue through angiogenesis may be an attractive treatment strategy for ischemic heart disease. To that end, novel insights in the genetic profile of endothelial cells and angiogenesis are required to fully understand the development of newly formed blood vessels. Here we used the complementary strength of mouse and zebrafish genomics to identify key regulators for vasculogenesis, angiogenesis and lymphangiogenesis. At different time points of murine vascular development, angioblasts (endothelial cell precursors) were sorted and their genetic profiles were compared. Genes that were up-regulated were further validated in zebrafish by *in situ* hybridization. Those genes exclusively or predominantly expressed in endothelial cells were knocked down by an *in vivo* anti-sense morpholino screen. Here we report the high throughput zebrafish screen which identified novel markers and potential regulators of zebrafish vascular development.

INTRODUCTION

The vascular system is the first functional organ that will develop in vertebrates. Vascular development is initiated with the differentiation of hemangioblasts into vascular endothelial cells in the mesoderm of the embryo. The first step of differentiation is the separation of hemangioblasts into angioblasts and hematopoietic cells. The angioblasts further develop into primitive blood vessels, whereas the hematopoietic cells will form all of the hematopoietic lineages. The complete process of differentiation of angioblasts towards the assembly of endothelial cells in vascular tubes is referred to as vasculogenesis (Risau and Flamme, 1995).

In mice, the first signs of intra-embryonic vasculogenesis are detected from embryonic day (E) 6.5. Numerous markers of mesoderm-derived hemangioblasts become apparent at this stage. Among the earliest markers of angioblasts are T cell acute lymphocytic leukemia-1 protein (*Tal1*) and Fetal liver kinase-1 (*Flk1*). These markers are co-expressed in the angioblasts (Drake et al., 1997; Shalaby et al., 1997). *Flk1* is not only one of the earliest markers for the hemangioblasts and endothelial cells (Shalaby et al., 1995); its expression is also required for proper blood vessel formation; *Flk1* deficient mice lack blood vessels and are embryonic lethal at E8.5 (Shalaby et al., 1995). During morphogenesis and assembly of vascular tubes, a set of markers follow each other rapidly in sequence of expression. Initially, Platelet endothelial cell adhesion molecule-1 (*PECAM-1, CD31*) (Baldwin et al., 1994) is expressed and followed by *CD34* (Fina et al., 1990; Young et al., 1995), vascular endothelial cadherin (*cadherin5, VE-cadherin*) (Lampugnani et al., 1992) and *Tie2* (Suri et al., 1996).

In zebrafish, similar to mice, the vasculature arises from endothelial cell precursors (angioblasts) from the lateral plate mesoderm (Figure 1A, B). Angioblasts give rise to the full set endothelial cells of all blood vessels. In the trunk, they assemble into a cord like structure and develop in primary tubes: the dorsal aorta (DA) and posterior cardinal vein (PCV). In addition, the primary anterior angioblasts assemble into the lateral dorsal aorta (LDA) and the aortic arches (AA) (Figure 1C). Then, when the heart is functional, blood is circulated through the trunk. After the primary circulation loop is established, the head vasculature is also provided with oxygen via the primitive internal carotid artery and returns via the primordial mid and hindbrain channels (PMBC/PHBC) back via the common cardinal vein (CCV) and the duct of Cuvier (DC). The initiation of newly formed blood vessels from existing vessels, angiogenesis, will give rise to the rest of the vasculature in zebrafish.

In the trunk, the newly formed blood vessels arise initially from single endothelial cells that sprout from the dorsal aorta exhibiting extensive filopodia activity (Figure 1C, right panel), and migrate from the dorsal aorta to the very dorsal side of the embryo, where it will give rise to the dorsal longitudinal anastomotic vessel (DLAV) (Figure 1C). The connecting vessel between the DLAV and DA, the intersegmental vessel (ISV) is then further developed and a lumen is established. After lumen formation, the intersegmental arteries become functional. After arterial angiogenesis

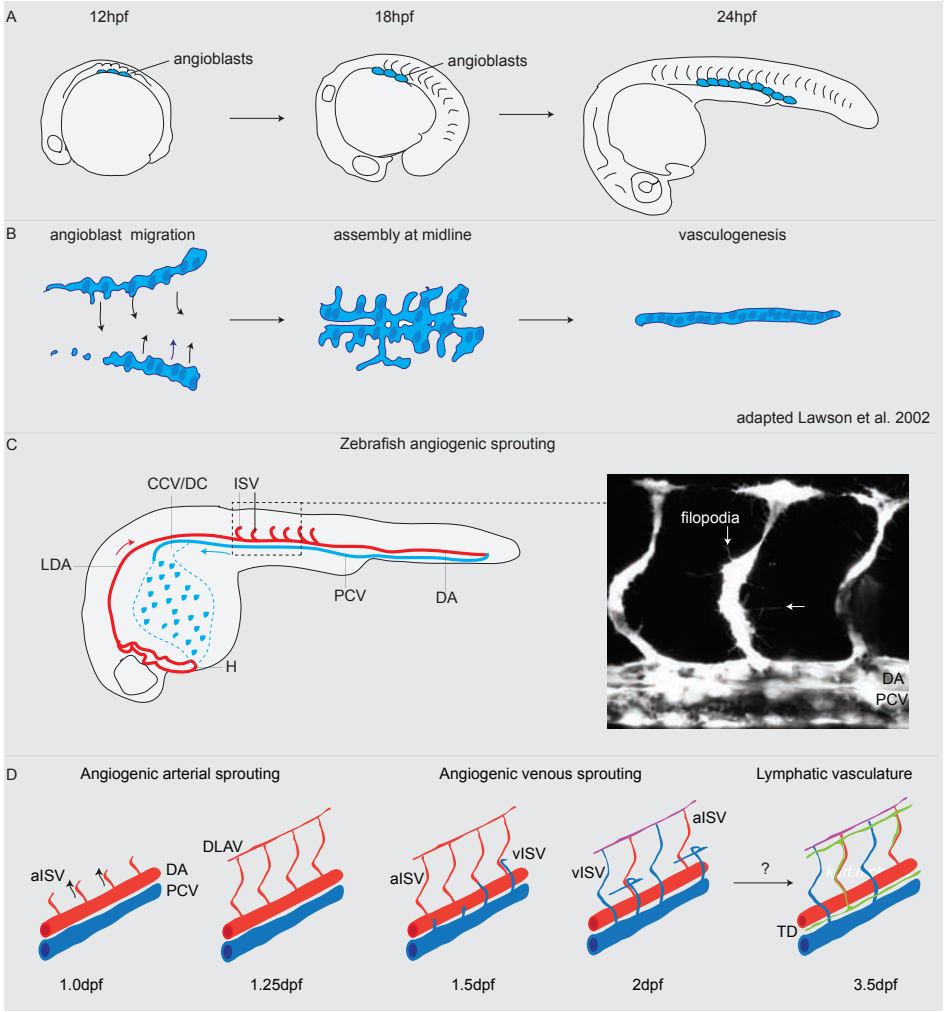


Figure 1: Zebrafish vasculogenesis, angiogenesis and lymphangiogenesis. A,B. Schematic representation of a 12 hpf, 18hpf and 26hpf embryo which represents the first cord-like structures by assembly of angioblasts in the embryo. C. Extensive filopodia activity during zebrafish trunk angiogenesis. D. Model representing the early key steps of zebrafish lymphangiogenesis. Dorsal Aorta (DA), posterior cardinal vein (PCV), Common cardinal vein (CCV), duct of Cuvier (DC), dorsal longitudinal anastomotic vessel (DLAV) , the venous (v) and arterial (a) intersegmental vessel (ISV), thoracic duct (TD).

has been established, the trunk vasculature is remodeled by secondary venous endothelial sprouting from the PCV (Isogai et al., 2001; Lawson and Weinstein, 2002b). Approximately half of these sprouts will connect to the existing arterial intersegmental vessels and become veins, thereby providing an equal distribution of arteries and veins. The other half was thought to give rise to the parachordal vessel (PAV) and it is has

been reported that this will further develop into the lymphatic vasculature (Figure 1D) (Covassin et al., 2009; Lawson and Weinstein, 2002a; Yaniv et al., 2006).

The main function of the zebrafish lymphatic vasculature is drainage of fluid from the interstitial space. Dye-uptake experiment injections and perfusion of the blood and lymphatic vasculature have elucidated that the blood vessels are at some point connected to the lymphatic vasculature (Yaniv et al., 2006). The major lymphatic vessel, the thoracic duct (TD) is situated between the DA and the PCV. In zebrafish, genes required for mammalian lymphatic development have been identified as well, suggesting that the genetic regulation of the lymphatic vasculature is genetically conserved throughout evolution. For example, it has been shown that *vegf-c* and the receptor *flt4* (VEGFR-3) are required for the development of the lymphatic vasculature (Kuchler et al., 2006) in zebrafish.

Identifying regulators of murine and zebrafish vascular and lymphatic development has already been shown to be efficient and productive (Covassin et al., 2009; De Maziere et al., 2008; Habeck et al., 2002; Sumanas and Lin, 2006; Wong et al., 2009). However, these studies were usually done in a one animal model. Since genetic regulators are likely to be evolutionary conserved (e.g. the vascular endothelial growth factors (Bussmann et al., 2008)), we aimed to use the complementary strength of both murine and zebrafish genetics to unravel novel genes of vascular development.

First, we sorted endothelial cells during crucial time points of murine vascular development and found several genes to be up-regulated and we validated for the presence of zebrafish orthologue. Furthermore, we performed large scale gene expression screen in zebrafish which resulted in the identification of several endothelial expressed genes. Only genes with unique expression patterns and uncharacterized function in the zebrafish were selected for an *in vivo* knockdown screen approach. A schematic representation of the genome wide genetic screening is depicted in Figure 2

RESULTS

To identify novel regulators (e.g. genes/proteins) of vascular development we collected mouse embryos at E10.5, E11.5 and 16.0. During these early time points (E10.5 and E11.5), loss of important regulators of vascular development will result in embryonic lethality, suggesting intensive vascular development. After dissociation of the embryos, cells were labeled with conjugated Flk1-specific antibodies. After fluorescent activated sorting, 1.5 % of the cells of the total population were found to be Flk1+ and we were able to obtain a 97% pure Flk1+ cell population after multiple rounds of sorting. We next compared the genetic profile during individual time-points in embryonic development by analyzing genes that were up-regulated at least two-fold (>1.95) in the Flk+ cells compared to Flk- population (Cheng et al., 2011). Over 30.000 transcripts were analyzed; 2161 transcripts were up-regulated at E10.5 and E11.5, and, when we excluded the genes that were also up-regulated at E16.0, we were left with 784

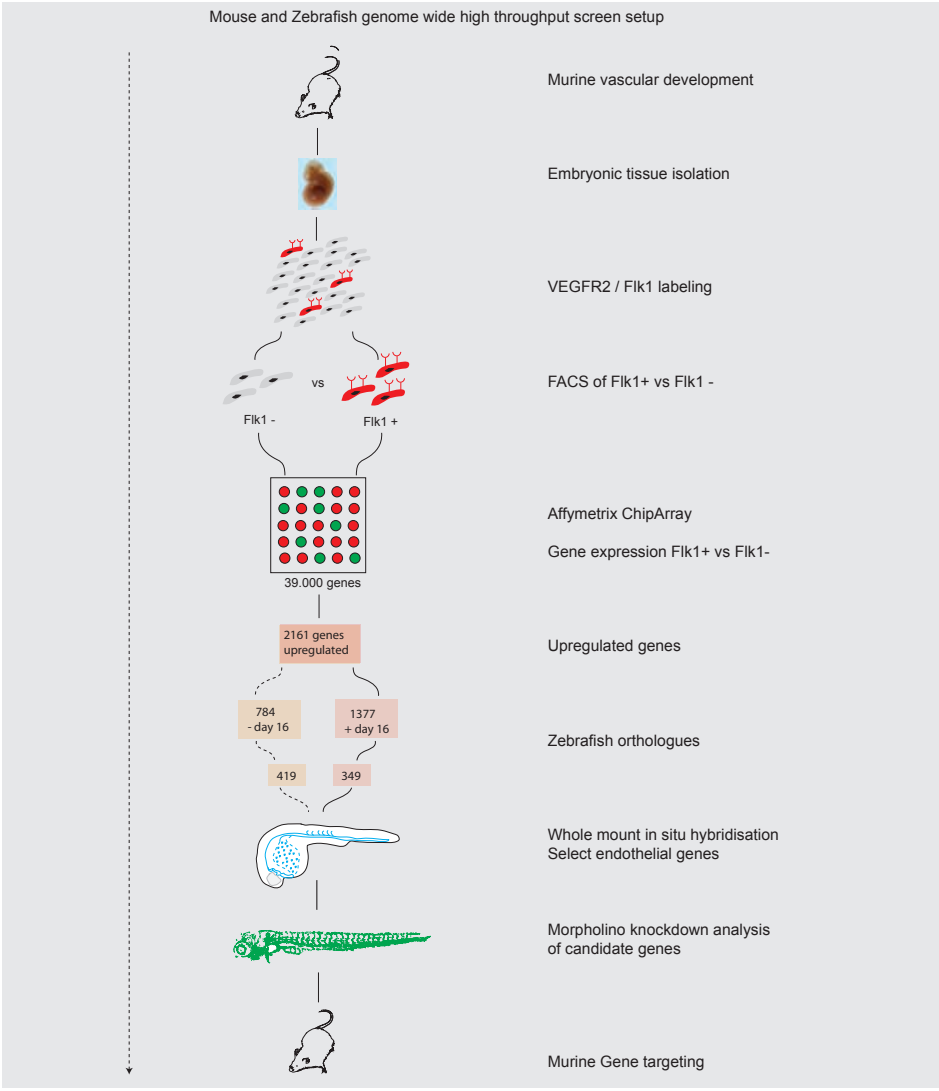


Figure 2: Graphical representation of the murine and zebrafish genome wide high throughput screen. Mouse embryos were collected and dissociated. Cells were labeled with conjugated Flk1-specific antibodies and PE antibodies for fluorescent activated cell sorting. Next, total RNA of both populations was isolated and used for microarray analysis. Genes that showed up-regulated expression levels were selected and validated to have e respective zebrafish orthologue and used for in situ hybridization. Finally, vascular-specific expressed genes were knocked down by morpholino injection in zebrafish to address the gene function. Ultimately, *in vitro* assays and gene targeting in mice will complement the conserved function of the respective genes.

transcripts. The exclusion of transcripts at E16.0 was done to enrich for genes that were specifically expressed early in development, which would correlate to their function at those time-points. The genes that were up-regulated 'early' at E10.5 and E11.5 which had already been implicated in vascular development are depicted in Table 1. For example, KDR/Flk1 was found to be up-regulated, indicating that our strategy for the isolation of endothelial expressed genes was effective.

Further validation of up-regulated transcripts was done in the zebrafish. This model organism exhibits rapid development of the cardio-vascular system within the first 24hrs, and is suitable for high throughput expression screening and subsequent gene knockdown analysis. The expression screen was done in two phases. First, we subtracted the genes that were expressed at late stages (E16.0) from those genes that were found to be up-regulated in early development (E10.5 and E11.5). In addition, we screened for genes that included the late (E16.0) time-point and hypothesized that without exclusion of E16.0, we would enrich for 'maintenance genes' and genes required for the establishment and maturation of the (lymphatic) vasculature.

Table 1. Regulators of vascular development

Table 1a. Key players at E10.5 + E11.5 and E16.0

Gene	type	function	reference
Flk1	receptor	vasculogenesis	Shalaby, 1995
Flt4	receptor	lymph)angiogenesis	Tammela, 2009
Lyve-1 (Xlkd1)	receptor	marker lymphatics	Luong, 2009
Vegfc	ligand	(lymph)angiogenesis	Karkkainen, 2004
Unc5b	receptor	angiogenesis	Eichmann, 2004

Table 1b. Key players at E10.5 + E11.5 without E16.0

Gene	type	function	reference
Flt1	receptor	angiogenesis	Ferrara, 2003
Lmo	transcription	hematopoesis	Landry, 2003
Ets	family transcription	angiogenesis	de Val and Black 2009
Tie1	receptor	angiogenesis	Thurston, 2003
Ang2	ligand	angiogenesis	Thurston, 2003
Nrp1, Nrp2	receptor	angiogenesis	Gerhardt, 2004
Dll4	ligand	angiogenesis	Gerhardt, 2009
Sox18, Sox7	transcription	specification	Herpers, 2009

Flk1+ versus *Flk1*- known regulators for vasculogenesis, angiogenesis and lymphangiogenesis. 1a: Including E16.0 in the microarray analysis resulted in more lymphatic markers and regulators such as *Vegf-c, Flt4 (Vegfr-3)* and *Lyve-1*. 1b: Excluding E16.0 resulted in an enrichment of angiogenesis markers and regulators such as *Ets1, Dll4, Sox18, Nrp1* and *Flt1*.

In this second phase, we excluded the 784 'early' genes from the 2161 transcripts and the remaining genes were used for further analysis and checked for the existence of a zebrafish orthologue using Biomart (www.ensemble.org). Only the murine transcripts which seemed to have a one-to-one orthologue in zebrafish (Zv6 and Zv7 genome assembly) were used. As a result, we could perform a high throughput expression screen for 419 'early' genes and '360' late genes. The result of the 419 'early' genetic screen is described elsewhere (Thesis Robert Herpers, 2010, ISBN: 978-90-9025220-9).

Table 2. Selected genes for morpholino knockdown

Morpholino	amount/range (ng)	phenotype	RT-PCR confirmed
Plvap MO-1	3, 6	no phenotype	no
Plvap MO-2	3, 6	no phenotype	no
Grrp1 MO-1	3, 6	no phenotype	yes
Grrp1 MO-2	3, 6	no phenotype	yes
Grrp1 MO-3	3, 6	toxic	
Stab1 MO-1	3, 6, 10	no phenotype	yes
Stab1 MO-2	3, 6, 10	no phenotype	yes
Stab2 MO-1	3.5, 7,0	no phenotype	no
Stab2 MO-2	3.5, 7.0	no phenotype	no
Stab2 MO-3	3.5, 7.0	no phenotype	no
Exoc3L MO-1	3, 6, 9	toxic	no
Exoc3L MO-2	3, 6, 9	no phenotype	no
Thsd1 MO-1	1, 2, 3, 6	hemorrhaging in head	yes
Thsd1 MO-2	1, 2, 3, 6	hemorrhaging	yes
Tnfaip8l1 MO-1 ATG/UTR	1, 2, 3, 6	phenotype	not possible
Tnfaip8l1 MO-2 UTR2 sense	1, 2, 3, 6, 8.33, 17	no phenotype	
Tnfaip8l1 MO-3 UTR3	4, 8, 12	phenotype	not possible
Tcfec MO-1	1.5, 3, 6	no phenotype	no
Tcfec MO-2	1, 3, 6	no phenotype	no
Tcfec MO-3	3, 6	no phenotype	no
Lgmn MO-1	1, 2, 3, 6	no phenotype	yes
Lgmn MO-2	1, 2, 3, 6	no phenotype	yes
Lgmn MO-3	0.5,1.0	no phenotype	not possible
Cyb5b MO-1	3,6	no phenotype	no
Cyb5b MO-2	3,6	no phenotype	no
Cyb5b MO-3	0.5, 1,0	phenotype, severe cell death	no

Candidate genes for morpholino knockdown. *Plvap, Grrp1, Stab1, Stab2, Exoc3L, Thsd1, Tnfaip8l1, TcFec, Lgmn* and *Cyb5b* were selected for morpholino knockdown. Amount of morpholino was titrated and appropriate concentration was used for in vivo analysis of the blood vasculature. Morpholinos were confirmed with RT-PCR if possible. Note: ATG and UTR morpholino cannot be confirmed by RT-PCR.

We next aimed to unravel the expression of 360 zebrafish transcripts by amplifying the largest translated exon from the zebrafish orthologues for probe generation. Out of 360 transcripts we could generate 315 probes and performed *in situ* hybridization at 16hpf, 24hpf and 32hpf with a minimum of 15 embryos per gene and per time-point. We then selected those genes that showed a novel zebrafish vascular expression pattern (Table 2).

We identified several endothelial expressed genes. *Plvap* (*pv-1*) or PlasmaLemmal Vesicle Associated Protein-1 is an endothelial-specific integral membrane glycoprotein, associated with endothelial cells and VEGF signaling (Hnasko et al., 2006a; Hnasko et al., 2006b; Stan et al., 1999) (Figure 3B, left panel). Strikingly, we found *Xlkd1/lyve-1* (Figure 3A, middle panel); a gene which is expressed abundantly on the surface of lymphatic vessels, early macrophages and lymph node sinus endothelial cells during early development, but is dispensable for lymphatic development (Banerji et al., 1999; Luong et al., 2009; Tripp et al., 2008; Wrobel et al., 2005). In addition, we also found *stabilin-1* and *stabilin-2*, two large transmembrane receptors with scavenging roles on endothelium and already shown to be implicated with the immune response (Karikoski et al., 2009; Kzhyshkowska et al., 2006a) (and reviewed in (Politz et al., 2002)).

Furthermore, a set of novel genes were found such as (1) *Thsd1*, a transmembrane molecule with thrombospondin module (*tmtsp*) is expressed in mouse hematopoietic stem and endothelial cells. The function of *thsd1* in mice and zebrafish is still unknown (Takayanagi et al., 2006) (Figure 3C, right panel). (2) *Grrp1,* an glycine/arginine rich protein with uncharacterized function. (3) *Tnfaip8l1* (tumor necrosis factor

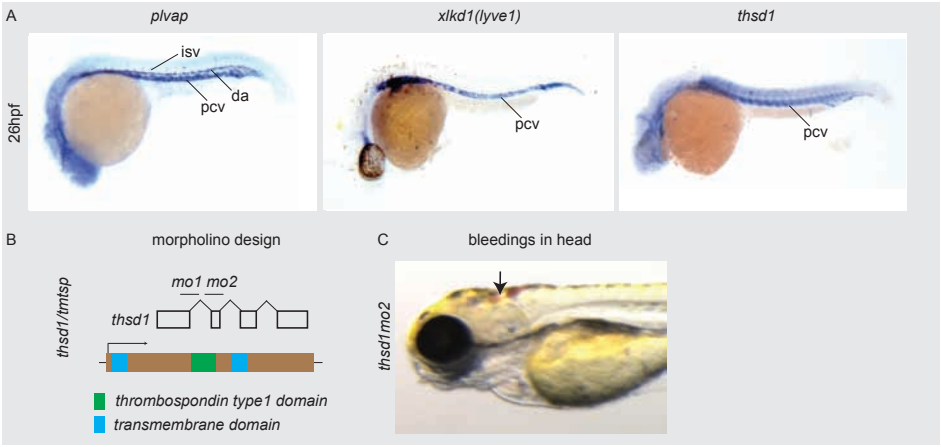


Figure 3: Expression and knock-down of endothelial expressed candidate genes. A. Lateral images of whole mount in situ hybridization for *plvap, xlkd1 (lyve1)* and *thsd1,* demonstrating expression in vascular endothelium at 26 hpf. B. Domain architecture of, and morpholino (MO) design for, the *thsd1* gene. Thsd1 contains a transmembrane domain and thrombospondin type-1 domain (tmtsp1). MO1 and MO2 disrupt splicing of exon-intron boundaries between exon1 and exon2 of *thsd1*. C. Loss of Thsd1 results in hemorrhaging in the head region (arrow) at 32 hpf.

alpha-induced protein 8-like 1), family members of this gene have been implicated in transcription factor NF-kappaB-inducible anti-apoptosis, but with unknown endothelial function, and (4) *Erg*, or Ets related gene, a member of the Ets family which contain a number of transcriptional activators and inhibitors of zebrafish angiogenesis (Brown et al., 2000; Ellett et al., 2009; Patterson and Patient, 2006).

Expression analysis was followed up by an *in vivo* knockdown screen using morpholinos (Sumanas and Larson, 2002). For each gene, multiple morpholino oligomers were designed and injected in one-cell stage embryos of transgenic embryos. We used the transgenic line Tg(*fli1a:eGFP*)^{y7}(Lawson and Weinstein, 2002b) which labels endothelial cells and screened for vascular defects in this system. An overview of the results of the knockdown screen is shown in Table 2.

Only knockdown of *thsd1* and *tnfaip* ulted in a vascular phenotype. Injectin two independent morpholinos targeting *Thsd1* resulted in hemorrhages in the head, whereas the overall vasculature appeared normal. In addition, two morpholinos targeting *tnfaip8* resulted in an angiogenic phenotype and will be described in more detail.

Tnfaip8 expression is restricted to the dorsal aorta and sprouting of intersegmental vessels from 22hpf to 28hpf (Figure 4A, and inset). Zebrafish *tnfaip8* was blocked by an ATG and UTR morpholino. Both morpholinos resulted in a similar phenotype; angiogenic sprouting was altered and guidance of intersegmental vessels was lost. As a result, the overall patterning of the vascular network was se affected (Figure 4B). To control for specificity, we generated a construct (*tnfaip8l1-RNA*) in which the full length version of *tnfaip8* was cloned. We hypothesized that the full length RNA construct would be expressed in all tissues and therefore might rescue the angiogenic phenotype. However, *Tnfaip8*-RNA was not able to rescue the angiogenic phenotype, whereas (low-dose) injection of the construct alone did not show any phenotype (Figure 4C).

Taken together, the high throughput cross species reverse genetics approach resulted in the identification of several novel angiogenic and lymphatic markers during zebrafish vascular development, whereas the insight into novel regulators of vascular development was limited.

DISCUSSION

High throughput screening for regulators of vascular development can be done in several ways. The classical forward genetic screens are designed to identify zebrafish with specific phenotypes. In these forward screens, zebrafish are mutagenized and crossed to transgenic *fli1a:*eGFP fish to generate F1 families. Then, F2 families were generated by incrossing F1 fish and mutant phenotypes were analyzed in F3 embryos. Vascular mutants are identified by an s of larvae at 24hpf for vasculogenesis, 48hpf for angiogenesis and 4dpf for lymhpangiogenesis. However, this classical forward genetic screening requires a lot of infrastructure and identifying the affected mutant

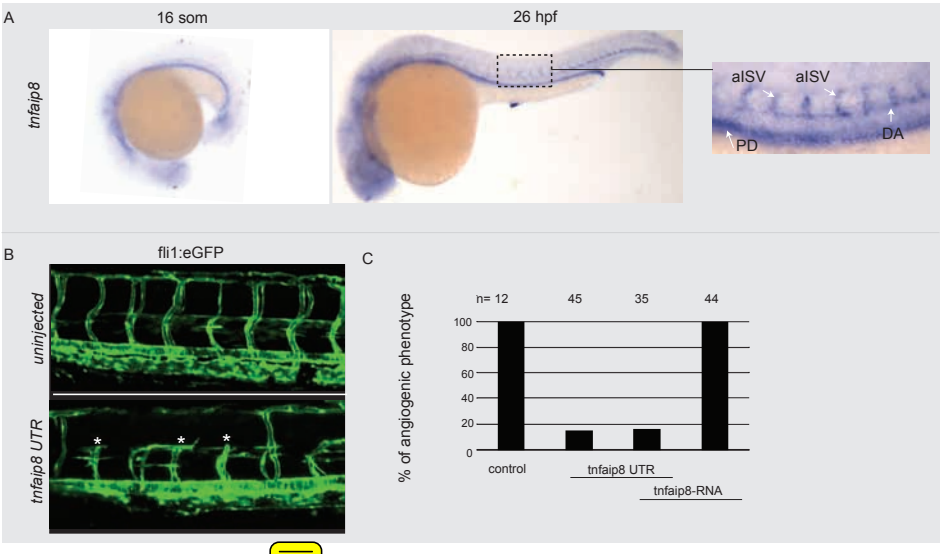


Figure 4: *Tnfaip8* and *Tnfaip8l1* expression pattern and knockdown analysis. A. In situ hybridization of *tnfaip8* during zebrafish development. At 16 somites, *tnfaip8* is expressed in the developing vasculature and the expression becomes restricted to arterial endothelium at 26 hpf. Inset showing expression in intersegmental vessels (ISV's), dorsal aorta (DA) and pronephric duct (PD). C. Domain architecture and morpholino (MO) design of *tnfaip8*. *Tnfaip8* encodes a predicted protein with 186 amino acids, containing a domain of unknown function (DUF). *Tnfaip8* is a one-exon gene and is targeted by an UTR morpholino, injection of which results in defective angiogenic arterial sprouting from the DA. Asterisk indicates defective sprouting angiogenesis in the zebrafish trunk at 48hpf. Vascular endothelium is visualized in the *Tg(fli1a:GFP)* line. E. *Tnfaip8-RNA* fails to rescue the angiogenic phenotype. At 48hpf, Tnfaip8 UTR morpholino results in 83% angiogenesis phenotype which cannot be rescued by RNA *Tnfaip8*.

gene can be time consuming. Therefore, reverse genetic screening for vascular specific genes in zebrafish can be faster and cheaper, when using the correct set up.

The high throughput, cross-species reverse genetic screen described here was successful to some extent. In search for genes that regulate the *de novo* synthesis of blood vessels, the first phase of screening (the 'early genes') resulted in a higher number of regulators of vascular development (e.g. *Dll4, Sox18, Sox7, Apelin, Agtrl1*) which can be explained by the exclusion of genes that are also up-regulated at later stages of development (E16.0). To directly find genes that are required for the assembly of angioblasts into cord-like structures, the usage of mouse embryos at E7.0 to E9.0 would probably be more suitable since vasculogenesis and angiogenesis is already initiated before E10.5.

Exploring the function of a vascular specific gene in zebrafish can be accomplished by injecting morpholino. In the case of *tnfaip8*, we followed this approach by blocking both the ATG and the UTR region of *tnfaip8* the gene. While we did observe a phenotype, we were unable to rescue the phenotype by full length *tnfaip8-RNA*. Since family members of *tnfaip8* are involved in apoptotic signaling, it is possible that

tnfaip8 is required for proper apoptotic balance in endothelial cells. The high dosage of *tnfaip8-RNA* at 1 cell stage could be affecting the general development of the embryo. Since the expression of *tnfaip8* is so restricted, there might be a tight controlled spatio temporal distribution of *tnfaip8* which is specifically required for the first sprouting of endothelial cells from the dorsal aorta. For now, it is still unclear what the exact function and in which stage of angiogenic development *tnfaip8* is required. In addition, it would better to be generate a stable mutant zebrafish line for the gene. Thus, for *tnfaip8*, we screened the Hubrecht & Sanger TILLING libraries, but did not identify a stable mutant zebrafish. In addition to the zebrafish work, a targeted gene inactivation approach has been initiated to unravel the function of *Tnfaip8l1* in mice. Further *in vitro* and *in vivo* work will hopefully elucidate the role of *tnfaip8* in vascular development.

MATERIAL AND METHODS

Mice

Embryos were collected from plugged FVB/N female mice and dissociated. Cells were incubated with DM (PBS, 10%FCS) with 0.12% Collagenase type 1 (Sigma, C0130) for 45 minutes at 37 degrees. Single cells were stained 1/50 PE anti mouse Flk1 antibody (BD, 555308). Hoechst was used to stain for dead cells. Two populations (Flk+/Hoechst vs Flk-/Hoechst) were sorted on a FACS Diva using two consecutive rounds to improve purity. High quality RNA was isolated via the Qiagen, RNeasy mini kit.

Microarray

Microarray analysis was performed at the Erasmus Medical Centre, Rotterdam. 5 ug of mRNA was used for dscDNA synthesis using the InVitrogen choice system. Biotin labeled RNA was made using the ENZO BioArray High Yield RNA transcript. 20ug of labeled cRNA was hybridized to the GeneChip Mouse Genome 430A& 430B 2.0 Arrays. Rosetta resolver was used to import calls and intensities, followed by normalization. Raw data were merged into MniViz and a threshold minimum was set at 30. Fold differences were calculated from log averages determined for the different experimental conditions. Correlating mouse gene transcripts with zebrafish orthologues was done using BioMart and the Ensembl database (http://www.ensembl.org), releases 38 and 39, 2006/2007.

Zebrafish

Fish were kept under standard husbandry conditions at the Hubrecht Institute. Transgenic line such as the Tg(kdrl:eGFP)^{s843} (Jin et al., 2005) and Tg(fli1a:eGFP)^{y7} (Lawson and Weinstein, 2002b) were used for visualization of the vasculature.

In situ probe generation and in situ hybridization

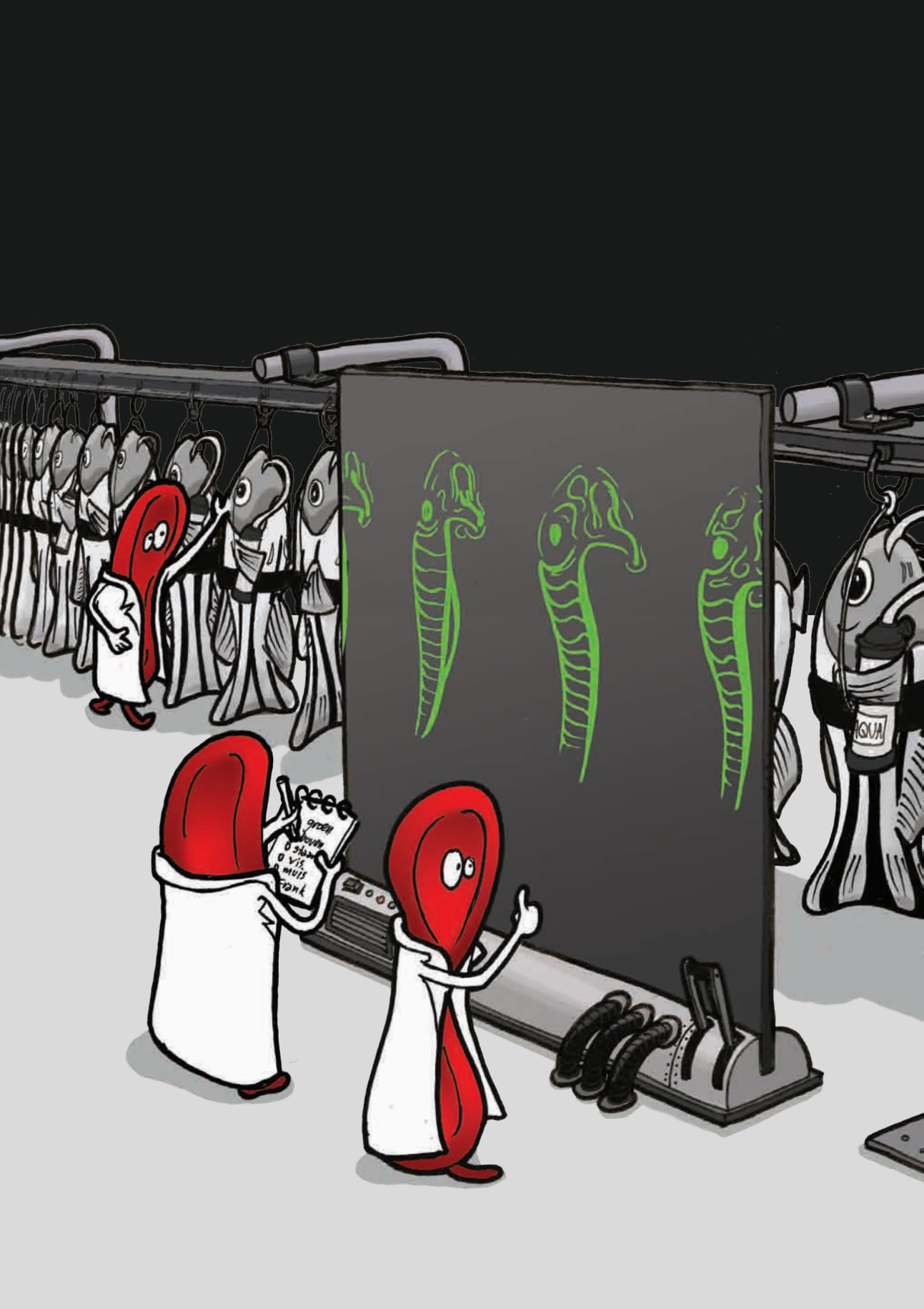
Genomic DNA was used for probe generation. Primers were designed of 150-250 base pair length covering the largest exon of each gene. Reverse primers were tagged with a T3 RNA polymerase promoter tail to follow in vitro transcription and anti-sense probe generation. Primers and sequences are listed in the supplementary material. In situ probes were DIG labeled using the Roche DIG labeling mix. Whole mount in situ hybridization was performed as previously described (Thisse and Thisse, 2008).

Morpholino injections

Morpholinos were obtained from GeneTools (www.gene-tools.com) and diluted in water containing 0.2% phenol red. Morpholinos were injected in one-cell stage embryos with 1-16ng in a maximum volume of 3nl. Sequences of morpholino are depicted in supplementary information.

Imaging

Zebrafish embryos were mounted in 0.25-1.0% low melting point agarose in a culture dish with a cover slip replacing the bottom. Imaging was performed with a Leica SP2 or SP5 confocal microscope using 10x, 20x and 40x objective.


AQUA

3

DEFICIENCY OF SCAVENGING RECEPTORS *STABILIN-1* AND *STABILIN-2* CAUSES POST-EMBRYONIC ANEMIA IN ZEBRAFISH

Frank L. Bos[1,2], Stefan Gorissen[2], Cornelia Veelken[2], Ewart de Bruijn[2], Henk van Roekel[2], Stefan Schulte-Merker[2] & Henricus J. Duckers[1]

1. Experimental Cardiology, Thoraxcenter, Erasmus University Medical Center, 's-Gravendijkwal 230, 3015 CE Rotterdam, The Netherlands
2. Hubrecht Institute-KNAW & University Medical Centre, Utrecht, Uppsalalaan 8 3584 CT, Utrecht, The Netherlands

Manuscript in preparation

SUMMARY

In mice, the lymphatic vasculature originates from veins and consists of specified lymphatic endothelial cells (LECs). In zebrafish, no functional assessment of the major scavenger receptors stabilin 1 and 2 is available at present. Here we report the identification and characterization of *stabilin-1* and *stabilin-2* in zebrafish development. To monitor the dynamics of venous endothelium *in vivo*, the promoter of *stabilin-1* was used to generate a stable *stabilin-1:YFP* transgenic line. We found that *stabilin-1* is expressed in venous endothelium and also labels all lymphatic structures until adulthood. To address the function of *stabilin* genes in development, we generated *stabilin-1* and *stabilin-2* mutant lines. These mutants are viable and apparently completely normal. Also, the double mutants develop initially in a normal fashion, with a normal blood and lymphatic vasculature. However, double mutants become progressively anemic. As a result, double mutants are lethal between 6 and 8 days post fertilization. Taken together, stabilins are expressed in venous and lymphatic endothelium but are dispensable for vascular development. However, expression of at least one of the stabilins is required for red blood cell homeostasis during zebrafish development.

INTRODUCTION

The blood vasculature is crucial for the exchange of gases, hormones and metabolites, whereas lymphatic vessels are important for proper fluid homeostasis and the regulation of the immune system. Both types of vessels consist of endothelial cells (ECs) and assemble into vascular networks. The importance of lymphatic vessels in normal tissue homeostasis is underscored by the fact that targeted disruption of the VEGF-C gene leads to lack of lymphatics vessels and as a result, mice die *in utero* displaying severe edema (Karkkainen et al., 2004). During embryogenesis, lineage tracing experiments in mice, frogs and zebrafish have shown that lymphatic endothelial cells (LECs) originate from veins (Hogan et al., 2009; Ny et al., 2005; Srinivasan et al., 2007). The lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) (Jackson et al., 2001) and Prospero homeobox 1 (Prox-1) (Oliver and Harvey, 2002) are indicative markers of LEC specification in the cardinal vein. Prox-1 positive and LYVE-1 positive LECs migrate away from veins in a process requiring VEGF-C and VEGFR-3 (Karkkainen et al., 2004; Kukk et al., 1996). Until now, *Prox1* (Wigle and Oliver, 1999), *Vegf-c* (Karkkainen et al., 2004) and *Vegfr-3* (Brice et al., 2005; Karkkainen et al., 2000) are the only known essential factors for murine lymphangiogenesis.

In zebrafish, the existence of a functional lymphatic system has recently been discovered (Hogan et al., 2009; Kuchler et al., 2006; Yaniv et al., 2006). Zebrafish lymphangiogenesis is the process of budding and migration of cells from (venous) endothelium towards lymphatic vessels. In zebrafish, venous sprouts either connect to the blood vascular endothelium (rendering arteries into veins) or gives rise to three distinct types of lymphatic vessels in larval zebrafish: dorsal longitudinal lymphatic vessel (DLLV), the intersegmental lymphatic vessels (ISLVs) and the major collecting vessel, the thoracic duct (TD) (Hogan et al., 2009; Isogai et al., 2001).

To fully understand the genetic regulation of lymphangiogenesis, we have performed a genome wide micro array analysis using an approach which combines the strength of murine and zebrafish vascular development. We isolated endothelial cells and compared their genetic profile during crucial time-point in murine lymphangiogenesis. Genes that were specifically up-regulated in endothelium were tested for having a regulatory role during vascular development in zebrafish. Here we report on the proteins *stabilin-1* and *stabilin-2*. We found that *stabilin-1* specifically marks venous and lymphatic vessels and structures. Furthermore, we found that both stabilins are not required for lymphangiogenesis but, interestingly, play a role in haematopoiesis.

RESULTS AND DISCUSSION

To identify novel markers of venous endothelial cells, we performed a genome wide micro array analysis during murine vascular development (data not shown). Since lymphangiogenesis is initiated at around E9.75 to E12.5 (Oliver and Srinivasan, 2010) and most of the lymphatic vasculature is formed at E16.5, we thus compared

their genetic profile at E10.5, E11.5 and E16.5. Next, genes that were specifically up-regulated in murine endothelial cells were translated to a zebrafish orthologue for fast and high throughput expression screening. To determine marker expression during key stages of zebrafish development, we first performed *in situ hybridization* of 315 genes and asked the question whether any were specifically expressed in endothelial cells. We observed several specific expression patterns in the developing zebrafish embryo from 26 hours post fertilization (hpf) to 50 hpf. We identified a type I membrane glycoprotein, extracellular link domain containing 1 (*xlkd1),* better known as lymphatic vessel endothelial hyaluronan receptor *lyve-1* (Figure 1A) (Flores et al., 2010). This result is compatible with previous findings which demonstrate that the mouse homologue *Xlkd1* or *Lyve-1* is expressed on lymphatic endothelial cells (LECs) and thus a marker for murine lymphatic development (Banerji et al., 1999). *Lyve-1* is involved in scavenging and transport of hyaluronan (HA), an important component of mesenchymal tissues. Whereas *Lyve-1* marks lymphatic endothelium early in mouse embryos, it is not required for lymphangiogenesis (Luong et al., 2009).

In addition to *Lyve-1,* we identified *Stabilin-1* and *Stabilin-2,* which belong to the same family of proteins with similar domain architecture (Politz et al., 2002; Schledzewski et al., 2006) (Figure 1A). *Stabilins* are large transmembrane receptors with fasciclin-like adhesion domains, epidermal growth factor (EGF) domains, one Link domain and an HA binding motif (Politz et al., 2002), and is expressed in most endothelial cells and the caudal haematopoietic tissues (Figure 1A, B). *Stabilin-1*, is also known as common lymphatic endothelial and vascular endothelial receptor-1 (*Clever-1),* is also a multifunctional glycoprotein. It is involved in linking signals from the extracellular environment to intracellular vesicular processes such as endocytosis (Kzhyshkowska et al., 2004; Kzhyshkowska et al., 2006b; Zhang et al., 2009). In addition to vesicular functions, *Stabilin-1* is involved in leukocyte trafficking and up-regulated upon inflammation of vascular endothelium (Palani et al., 2011; Salmi et al., 2004).

Since lymphatics originate from venous endothelium (Srinivasan et al., 2007; Srinivasan et al., 2010; Wigle et al., 2002), using *lyve-1* and *stabilin* as venous markers might provide more insight in the dynamics of venous endothelial cell behavior and the development of the lymphatics *in vivo*. Therefore, we aimed to develop transgenic zebrafish lines for *lyve-1, stabilin-1* and *stabilin-2*. The promoter region of each individual gene was used to drive expression of *YFP*. However, we were only able to successfully generate a *stabilin-1:YFP* transgenic line.

To unravel and define more precisely the expression of *stabilin-1:YFP* during zebrafish development, we characterized the expression at 7, 14 and 21 dpf. Strikingly, *stabilin1:YFP* expression becomes restricted to venous endothelium but importantly, labels lymphatic structures (Fig 1C, D). At 7dpf and 14dpf, *stabilin-1* is expressed in the trunk lymphatics, which consist of the intersegmental lymphatic vessel (ISLV), dorsal lateral lymphatic vessel (DLLV) and thoracic duct (TD) (Figure 1C, D). In addition, *stabilin-1* is also expressed in facial lymphatic vessels (FL), jugular lymphatics (JL) and pectorial lymphatics (PL) (Figure 1E). All these lymphatic structures drain towards the common cardinal vein (CCV) (Fig 1E) (Yaniv et al., 2006). In addition, the longitudinal

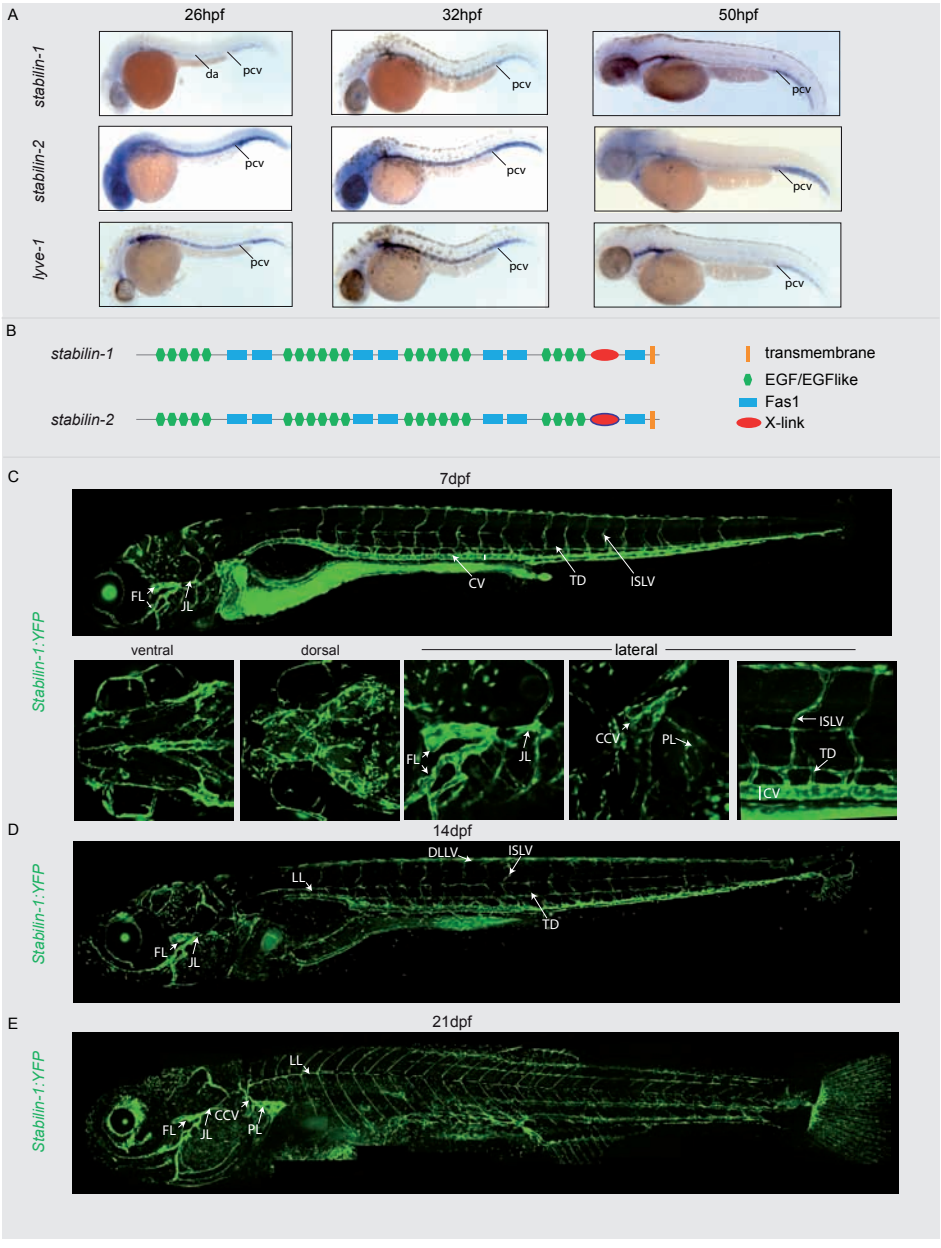


Figure 1: Stabilin genes are expressed in venous and lymphatic endothelium. A. *In situ* mRNA expression of *Lyve-1*, *Stabilin-1* and *Stabilin-2* in zebrafish development at 26hpf, 32hpf and 50hpf. *Stabilin-1* and *Stabilin-2* are both expressed in vascular endothelium. At 50 hpf, the expression becomes more restricted to venous endothelium. B. Domain architecture of *Stabilin-1* and *Stabilin-2*. C. *Stabilin-YFP* transgenic line which labels facial lymphatics (FL), jugular lymphatics (JL), cardinal vein (CV), intersegmental lymphatic vessels (ISLV) and thoracic duct (TD). Higher magnification images of ventral, dorsal head and lymphatic endothelium, common cardinal vein (CCV) and pectoral lymphatics (PL). *Stabilin1:YFP* at 14 days (D) and at 21 days (E) marks adult lymphatic vessels.

lateral lymphatics (LL) are visible at around 14dpf (Figure 1D, E). These results indicate that *stabilin-1* labels venous and lymphatic endothelium from embryonic to adult zebrafish development.

Previous work on zebrafish lymphatic development was limited due to the lack of specific transgenic lines. The commonly used line *(fli1a:egfp)*^{y7}, which labels all endothelial cells, is not restricted to venous endothelium and lymphatic vessels. Here, we show that *stabilin-1:YFP* labels all structures which were previously identified by lymphangiography and dye injections (Kuchler et al., 2006; Yaniv et al., 2006). At 5 weeks, we could easily identify the CCV, JL, FL and PL in the *stabilin-1:YFP* line. To functionally test the drainage capacity off *stabilin-1:YFP* structures, we injected fluorescent microspheres near *stabilin1:YFP*+ regions. The LL and ISLV were indeed capable of taking up the microspheres from the interstitium. In addition, the CCV (Figure 2B, top panels) was filled with dye, when injections were carried out near the FL and JL, indicating that *stabilin-1*$^{+}$ structures label functional lymphatics in the head region (Figure 2B).

The expression of *stabilin-1* in venous and lymphatic endothelium would possibly suggest a functional role in these tissues. To address this, we generated stable mutants of *stabilin-1* and *stabilin-2* by TILLING (Targeting Induced Local Lesions IN Genomes) (Wienholds et al., 2003). For *stabilin-1*, we found a T to A transversion at amino acid 270, changing a cysteine into a premature stop codon (*stab1*^{C270X}) (Figure 3A). In addition, we identified a mutant of *stabilin-2* and found a C to T transversion at amino acid 266, changing an arginine to a premature stop (*stab2*^{R266X}) (Figure 3A). We next checked for expression of *stabilin-1* and *stabilin-2*, respectively, in both mutants at 26hpf by in situ hybridization. For *stabilin-1*, mRNA levels were severely reduced or absent in *stab1*^{C270X} probably due to non-sense mediated decay of the mutant mRNA. The expression of *stabilin-2* mRNA appeared unaffected in *stab2*^{R266X} mutants (Figure 3B).

To further address the function of *stabilin* in zebrafish development, we analyzed in-crosses of *stabilin* mutant carriers. Both *stab1*^{C270X} and *stab2*^{R266X} were homozygous viable and showed no abnormalities during (vascular) development. This suggests that either *stabilin-1* or *stabilin-2* do not have essential roles in embryonic development, or that there is functional redundancy between both genes. To test experimentally if stabilins have redundant roles in development, we raised an incross of *stabilin-1*$^{+/-}$ *stabilin-2*$^{+/-}$ double heterozygote carriers to adulthood. Double homozygous mutants (*stabilin1*$^{-/-}$/ *stabilin2* $^{-/-}$) are apparently healthy and develop normally until 5dpf, but do not survive to adulthood (Figure 3C). This indicates a requirement for at least one of the *stabilin* genes for proper larval development and thus at least partial functional redundancy of the two genes. By monitoring the survival rate of double mutants we found that *stabilin1*$^{-/-}$/ *stabilin2*$^{-/-}$ are lethal between 6 dpf and 14 dpf (Figure 3D).

To determine the cause of death of *stabilin1*$^{-/-}$/*stabilin2*$^{-/-}$mutants, we performed a detailed analysis of the vasculature between 4dpf and 7dpf. Strikingly, the overall patterning of the blood and lymphatic vasculature appeared unaffected. All blood vascular structures such as the DA, PCV, ISV and lymphatic vessels (DLLV, TD and ISLV) were present, indicating normal vascular development (Figure 4A). In

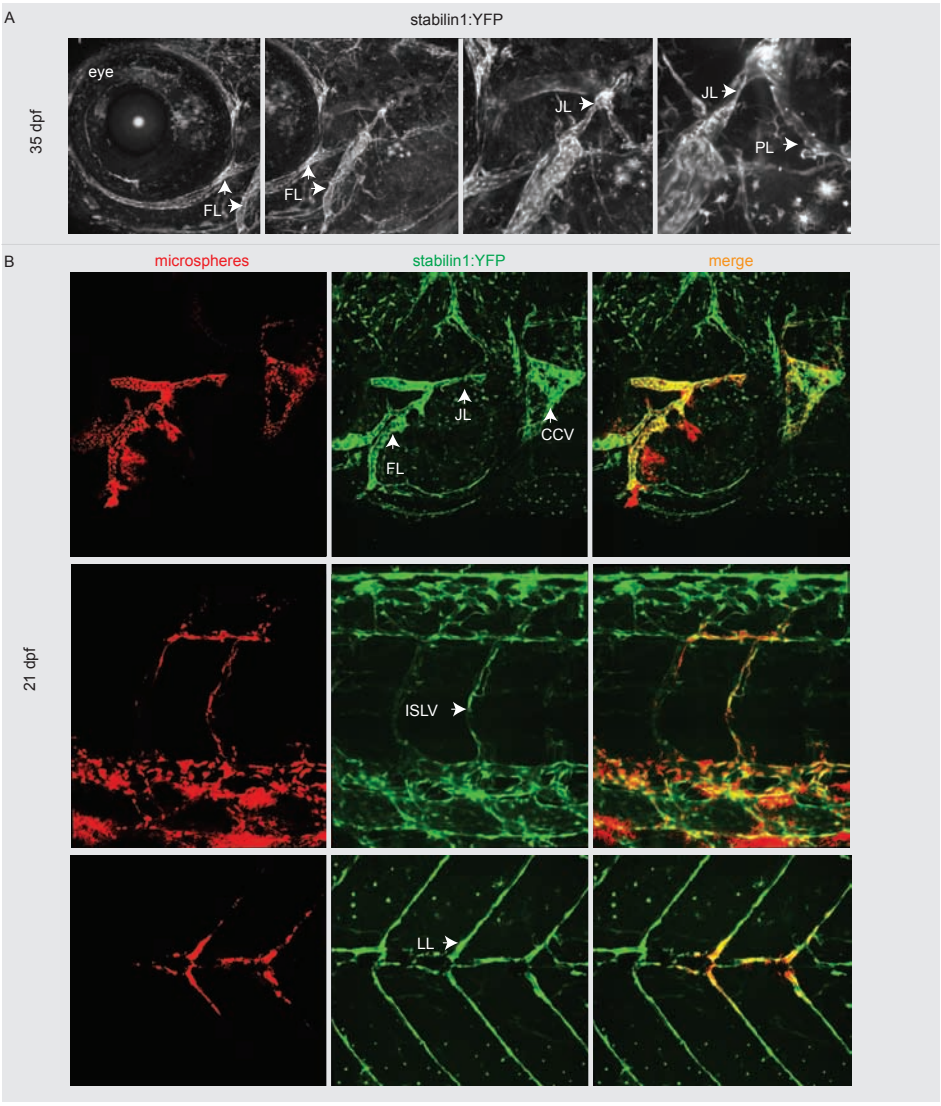


Figure 2: Stabilin-1 marks adult lymphatic structures. A. High resolution multiphoton imaging of a *stabilin1:YFP* larva at 35dpf revealing zebrafish facial lymphatics (FL), jugular lymphatics (JL) and pectoral lymphatics (PL). B. Fluorescent microspheres (red) were injected near lymphatic structures (green); lateral lymphatics (LL), intersegmental lymphatic vessels (ISLV), facial lymphatics (FL) and jugular lymphatics. Overlay highlighting lymphatic structures in orange.

addition, angiographies revealed that the *stabilin1*$^{-/-}$/ *stabilin2*$^{-/-}$ embryos still have a normal circulation (Figure 4B). However, within the circulation, the amount of erythrocytes in the blood stream was severely reduced at 5 dpf (Figure 4B, arrows). Monitoring the circulating erythrocytes between 4dpf and 6dpf revealed that flow is normal, but the total amount of erythrocytes progressively diminishes (Figure 4B,

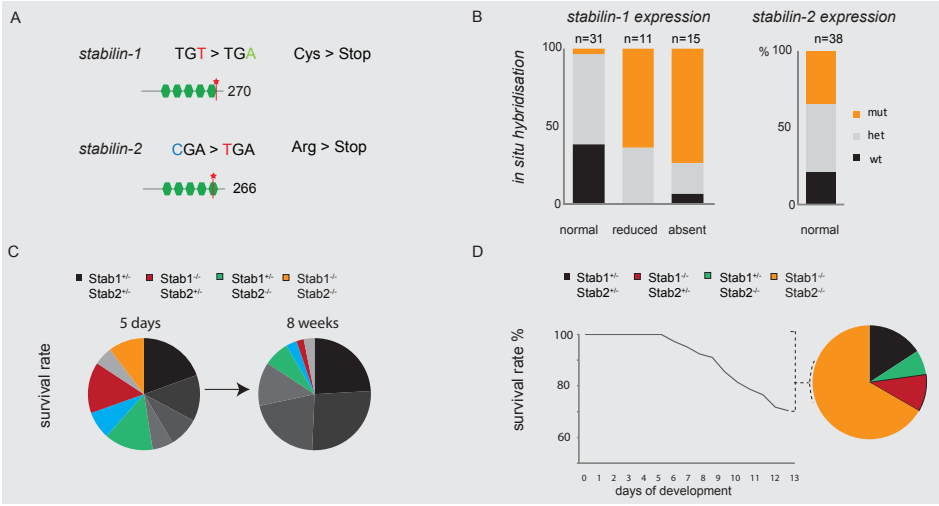


Figure 3: Stabilin double mutants are embryonic lethal. A. *Stabilin-1* and *stabilin-2* mutants. *Stab1*^{C270X} results in a T to A transversion at amino acid 270, changing a cysteine into a stop codon. *Stab2*^{R266X} results in a C to T transversion at amino acid 266, changing an arginine into a stop codon. B. Quantification of *in situ* hybridization of *stabilin-1* and *stabilin-2* showing a reduced or absent expression of *stabilin-1* in *Stab1*^{C270X}, whereas *stabilin-2* expression is retained in *Stab2*^{R266X}. C. *Stabilin1*$^{-/-}$*/stabilin2*$^{-/-}$ (orange) are lethal, revealed by the presence of double mutants at 5dpf, but are do not survive until 8 weeks. D. Survival curve of *stabilin1*$^{-/-}$*/stabilin2*$^{-/-}$ and siblings. Embryos were monitored daily and the majority of dead embryos were genotyped as *stabilin1*$^{-/-}$*/stabilin2*$^{-/-}$.

supplementary movie 1-4). To further validate the loss of red blood cells, we performed an *O-dianisidine* staining which detects hemoglobin in erythrocytes. Erythrocytes are hence stained brown, but in *stabilin1*$^{-/-}$*/ stabilin2*$^{-/-}$ mutants, staining was reduced or absent (Figure 4C). In addition, we observed that the shape and function of the heart in anemic double mutants eventually beats irregularly (Figure 4D), probably due to the anemia. Therefore, anemia is likely to be the cause of death of *stabilin* double mutants. Taken together, these data suggest that loss of *stabilin-1* and *stabilin-2* results in loss of erythrocytes in circulation and then an abnormal heart function after 5dpf.

Evidence from mice and *in vitro* work demonstrates the possible function of *stabilins* in development (Schledzewski et al., 2011). Recent work has shown that stabilins are required for proper tissue homeostasis, which involves the clearance of waste products by the endothelium before it can cause harm to surrounding tissues. Most of this clearance is done by the liver sinusoidal endothelial cells (LSEC). LSEC express several receptors which include *Stabilin-1* and *Stabilin-2*. Genetic evidence from mice lacking *Stabilin-1* or *Stabilin-2* revealed that single mutants are phenotypically normal, which resembles the observation with our mutant zebrafish. However, mice lacking both *Stabilin-1* and *Stabilin-2* are also lethal and harbor severe fibrosis of kidney structures, whereas the liver showed only mild fibrosis (Schledzewski et al., 2011). In addition, it was shown that the clearance of the TGF-β family member growth differentiation

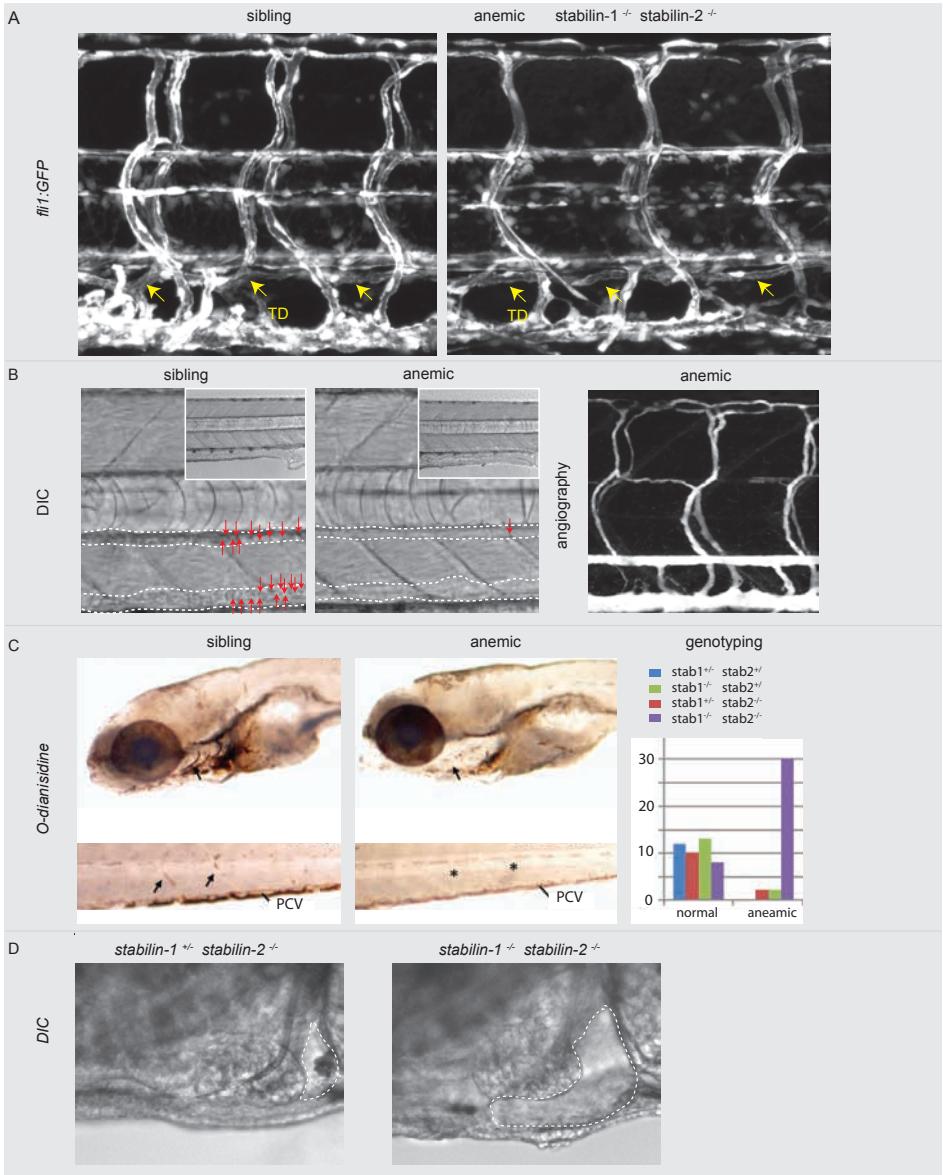


Figure 4: *Stabilin* double mutants become anemic. A. Confocal image of a Tg(*fli1a:GFP)* embryo representing normal blood- and lymphatic vascular structures at 5dpf. B. DIC images of anemic embryo compared to sibling embryo at 5dpf. Dashed lines indicated the dorsal aorta (upper) and posterior cardinal vein (lower). Arrows indicate the erythrocytes in a small region in circulation. Note that in anemic embryos, the amount of erythrocytes is severely diminished. Angiography of an anemic embryo with apparently normally lumenized vessels and circulation (left panel). C. *O-dianisidine* staining of mutants confirms a reduction in the amount of total erythrocytes in the blood stream. The bars demonstrates that anemic zebrafish (based on *O-dianisidine)* were mostly genotyped as *stabilin1*$^{-/-}$*/stabilin2*$^{-/-}$ or lacked at least three of the four stabilin alleles. D. DIC image of an abnormal heart in a *stabilin1*$^{-/-}$*/stabilin2*$^{-/-}$mutant embryo compared to siblings.

factor 15 (GDF-15) is impaired in mice lacking *Stabilin-1* and *Stabilin-2*. GDF-15 is one of the ligands for *Stabilin-1* and *Stabilin-2*, therefore it could be possible that clearance of this growth factor is required for proper erythrocyte homeostasis. Strikingly, GDF-15 is involved in late onset erythropoiesis and is found to be elevated in serum levels of patients suffering from thalassemia syndromes (Tanno et al., 2010; Whitman, 1998). Thallassemia is characterized by ineffective erythropoiesis which is caused by increased apoptosis or erythrocytes due to imbalance in globin chain arrangement (Schrier, 2002). Therefore, the anemic phenotype in zebrafish can be explained by a reduced uptake of GDF-15 by the scavenging receptors *stabilin-1* and *stabilin-2*, thereby resulting in an increased level of GDF-15 in serum levels and increased apoptosis of erythrocytes, but this remains to be elucidated.

In zebrafish, haematopoiesis (and thereby the genesis of erythrocytes) occurs in two separate waves. Between 24hpf and 48hpf, primitive haematopoiesis is initiated and is taken over by definitive haematopoiesis at 5dpf (de Jong and Zon, 2005). In addition, zebrafish definitive haematopoiesis is dependent on the expression of *Runx1*. The *Runx-1*+ haematopoietic stem cells (HSCs) emerge from the dorsal aorta, through the sub-aortic space, towards the axial vein in the blood stream by a process called the endothelial haematopoietic transition (Kissa and Herbomel, 2010; Kissa et al., 2008). Since HSCs have been shown to move from the dorsal aorta towards the axial vein (Kissa et al., 2008) and since stabilins are expressed as scavenging receptors on venous endothelium, it would be informative to test whether stabilins have a role in the *Runx1*+ HSCs endothelial haematopoietic transition.

Stabilin double mutants apparently undergo normal primitive haematopoiesis, but defective definitive haematopoiesis, resulting in an overall decrease in number of erythrocytes. These anemic phenotypes have been characterized before and several bloodless mutants have been isolated by forward genetics screening. Theses bloodless mutants are subdivided in either mutants with no red blood cells, progressive anemia and hypochromic anemia (decreased level of hemoglobin)(de Jong and Zon, 2005). However, none of these mutants have been linked genetically to stabilin genes, compatible with the redundant role of the two genes in this process.

Taken together, we demonstrate for the first time a role for stabilins in haematopoiesis in zebrafish. Since the double knockout mice are lethal due to severe fibrosis of kidney structures, we suggest that this could be due to severe anemia which results in organ failure and lethality. However, future research is required to understand the exact role of stabilin genes in the onset of anemia and definitive haematopoiesis.

MATERIAL AND METHODS

Zebrafish

Fish were kept under standard husbandry conditions at the Hubrecht Institute. Transgenic line such as the Tg(kdrl:eGFP)^{s843} (Jin et al., 2005) and Tg(fli1a:eGFP)^{y7} (Lawson and

Weinstein, 2002b) were used for visualization of the vasculature. Detailed information of the generation of Tg(Flt1^{enh}RFP), Tg(Stabilin-1:YFP) and Tg(SAGG27FFC:UAS:GFP) can be found in chapters 3 and 4. Primer information and *stabilin-1* and *stabilin-2* TILLING procedures are listed in the supplementary Excel files.

Morpholino injections & In situ hybridization

Morpholinos were obtained from Gene Tools (www.gene-tools.com) and diluted in water containing 0.2% phenol red. Morpholinos were injected at the one-cell stage (1-16ng) in a maximum volume of 3nl. Sequences of morpholino are depicted in supplementary information. In situ probes were DIG labeled using the Roche DIG labeling mix. Whole mount in situ hybridization was performed as previously described (Thisse and Thisse, 2008).

O-dianisidine staining

To detect hemoglobin, blood cells or anesthetized embryos were stained with 0.8 mg/ml *o*-dianisidine (Sigma-Aldrich) in 40% EtOH, 0.01M NaAc pH 5.2 and 2% H2O2 for 15 min in the dark.

Imaging

Zebrafish embryos were mounted in 0.25-1.0% low melting point agarose in a culture dish with a cover slip replacing the bottom. Imaging was performed with a Leica SP2 or SP5 confocal microscope using 10x, 20x and 40x objective.

SUPPLEMENTARY MATERIALS

Supplementary movie 1: High speed time-lapse movie of a 5 dpf sibling embryo, revealing wildtype amounts of circulating erythrocytes in the blood stream.

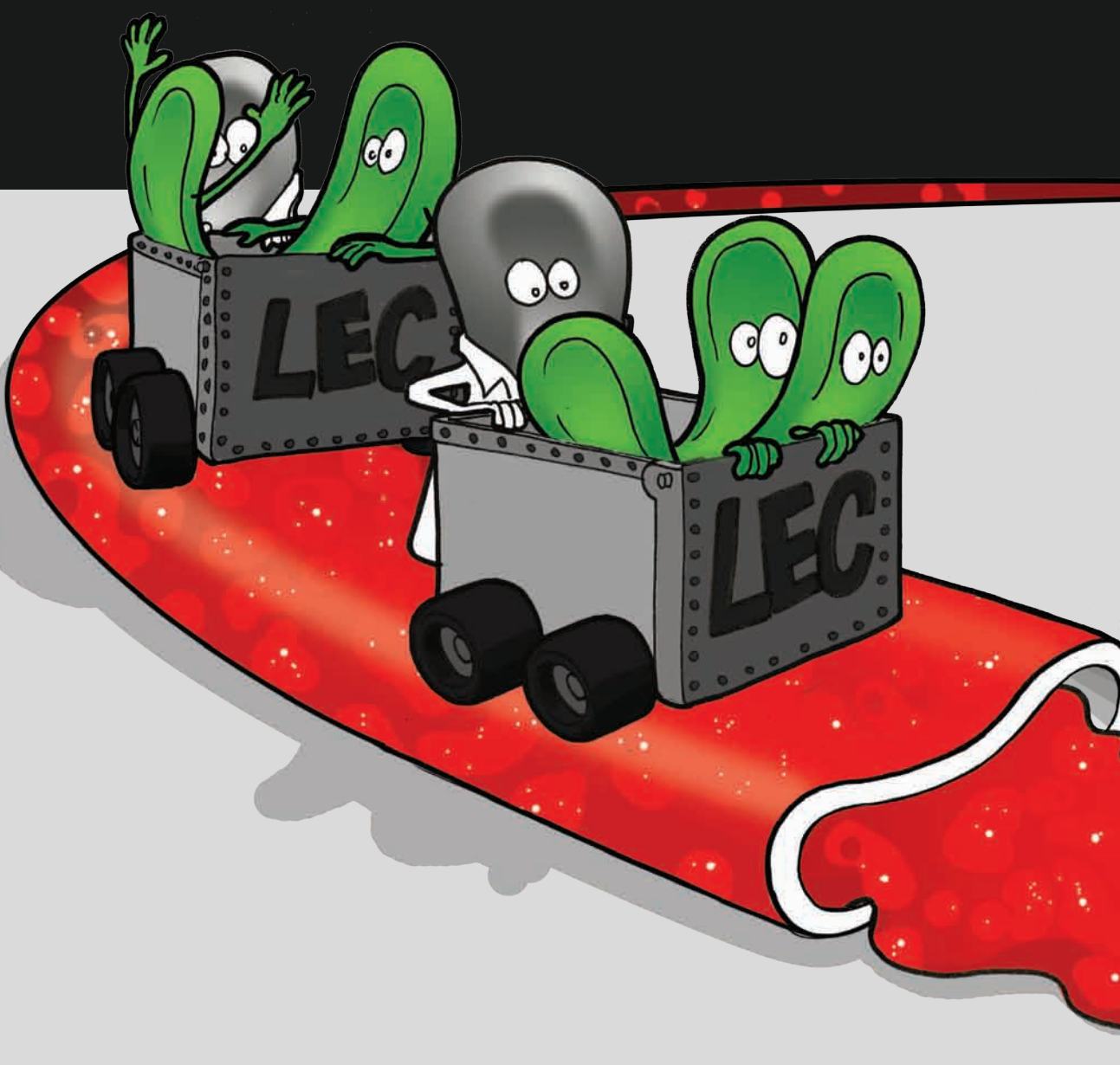
Supplementary movie 2: High speed time-lapse imaging of a 5 dpf *stabilin1$^{-/-}$/stabilin2$^{-/-}$ mutant, revealing the absence of erythrocytes in the blood stream.*

Supplementary movie 3: High speed time-lapse imaging of a 5 dpf sibling embryo revealing normal amount of circulating erythrocytes in the blood stream and through the heart.

Supplementary movie 4: High speed time-lapse imaging of a 5 dpf stabilin1$^{-/-}$/stabilin2$^{-/-}$ heart revealing the absence of erythrocytes in the blood stream and through the heart.

4

ARTERIES PROVIDE ESSENTIAL GUIDANCE CUES FOR LYMPHATIC ENDOTHELIAL CELLS IN THE ZEBRAFISH TRUNK



Frank L. Bos[1,2*], Jeroen Bussmann[1*], Akihiro Urasaki[3], Koichi Kawakami[3,4], Henricus J. Duckers[2] and Stefan Schulte-Merker[1]

1. Hubrecht Institute – KNAW University Medical Centre, and Centre for Biomedical Genetics, Uppsalalaan 8, 3584 CT Utrecht, the Netherlands.
2. Molecular Cardiology Laboratory, Experimental Cardiology, Thoraxcenter, Erasmus University Medical Centre Rotterdam, 3015 CE Rotterdam, the Netherlands.
3. Division of Molecular and Developmental Biology, National Institute of Genetics, 1111 Yata, Mishima, Shizuoka 411-8540, Japan.
4. Department of Genetics, the Graduate University for Advanced Studies (SOKENDAI), 1111 Yata, Mishima, Shizuoka, 411-8540, Japan.

* Authors contributed equally

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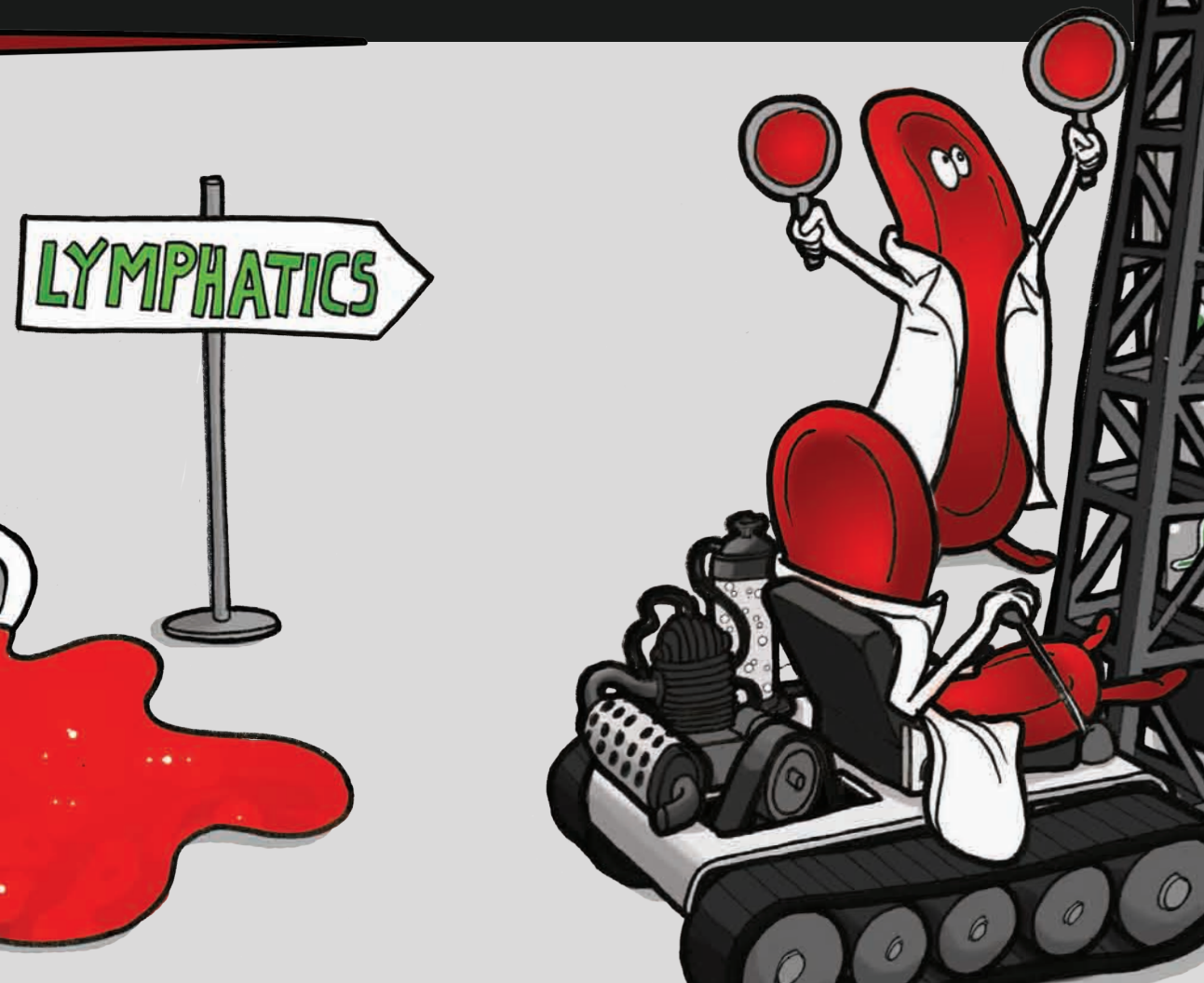


SUMMARY

The endothelial cells of the vertebrate lymphatic system assemble into complex networks, but local cues that guide the migration of this distinct set of cells are currently unknown. As a model for lymphatic patterning, we have studied the simple vascular network of the zebrafish trunk consisting of three types of lymphatic vessels that develop in close connection with the blood vasculature. We have generated transgenic lines that allow us to distinguish between arterial, venous and lymphatic endothelial cells (LECs) within a single zebrafish embryo. We found that LECs migrate exclusively along arteries in a manner that suggests that arterial endothelial cells serve as the LEC migratory substrate. In the absence of intersegmental arteries, LEC migration in the trunk is blocked. Our data therefore demonstrate a crucial role for arteries in LEC guidance.

INTRODUCTION

The vertebrate blood vessels are crucial for the exchange of gases, hormones and metabolites, whereas lymphatic vessels are important in the regulation of the immune system and fluid homeostasis. Both types of vessels assemble into complex vascular networks that display a remarkable degree of conservation in pattern formation (Weinstein, 1999), but how lymphatic endothelial cells (LECs) are guided and go on to form the lymphatic vascular system is still unclear. It has been noted that collecting, but not capillary, lymphatic vessels frequently align with blood vessels (Alitalo et al., 2005) but whether this indicates common guidance factors is not known.

The existence of a lymphatic system in zebrafish has recently been documented (Hogan et al., 2009; Kuchler et al., 2006; Yaniv et al., 2006). Here, we have studied the formation of the lymphatic network in the zebrafish trunk in more detail. The lymphatic network arises from cells that directly bud from the posterior cardinal vein (PCV) to the horizontal myoseptum between 1.5 and 2 days post fertilization (dpf). Some of these cells reconnect to the primary arterial sprouts and remodel into veins (Isogai et al., 2001). The other cells do not connect, continue to migrate dorsally and constitute so-called parachordal lymphangioblasts (PLs) that align along the horizontal myoseptum (Hogan et al., 2009) before they start migrating dorsally or ventrally (then referred to as LECs) at 3 dpf. These migratory cells give rise to three distinct types of lymphatic vessels at 4 dpf: the thoracic duct (TD), the intersegmental lymphatic vessels (ISLVs) tightly abutting the intersegmental blood vessels, and the dorsal longitudinal lymphatic vessel (DLLV) (Hogan et al., 2009).

Given the simplicity of this network and the amenability of the zebrafish embryo to *in vivo* imaging, this lymphatic network provides an ideal model in which to study lymphatic endothelial cell migration and patterning in a living embryo. Similar to the collecting lymphatic vessels in mammals this network forms in close association with the blood vasculature (Alitalo et al., 2005). Using newly established transgenic lines that allow the direct visualization of arteries, veins and lymphatic vessels within a single embryo, we found a striking tendency of ISLVs to align with arterial intersegmental vessels (aISVs), but not venous intersegmental vessels (vISVs). By analyzing embryos with defective arterial patterning, we show that aISVs are required for LEC migration and provide crucial guidance cues for LECs in the zebrafish trunk.

RESULTS AND DISCUSSION

Transgenic labeling of arteries, veins and lymphatic vessels

To study lymphatic patterning in relation to the blood vascular network, we aimed to develop a transgenic line in which arteries, veins and lymphatic vessels could be distinguished in a single living embryo. We have previously identified a transgenic line (*flt1*^{BAC}*:YFP*) in which arterial endothelial cells are specifically labeled (Hogan et al., 2009). Although *flt1*^{BAC}*:YFP* is an early marker for arterial endothelial cells, reporter

gene expression becomes upregulated in all blood endothelial cells after the onset of blood flow, making it less useful to visualize arterio-venous differentiation during the stages when lymphatic network formation occurs. To unravel a more general marker for zebrafish arteries, we used an *in silico* approach to identify potential regulatory elements necessary for early *flt1* expression in arterial endothelial cells. This analysis combined conserved non-coding elements and enhancers within the proximal region of the *flt1* promoter (for detailed descriptions, see the supplementary material). Two conserved elements were found to drive arterial expression and in combination with the *flt1* proximal promoter, this 0.8 kb promoter-enhancer construct was used to generate the *flt1*^{enh}*:RFP* transgenic line (Figure 1A, B) Within the trunk vascular network, aISVs display high RFP expression (*flt1*^{enh}*:RFP*^{high}) whereas vISVs display low RFP expression (*flt1*^{enh}*:RFP*^{low}). In lymphatic vessels, RFP expression is undetectable (*flt1*^{enh}*:RFP*^{neg}). The expression of *flt1*^{enh}*:RFP* was a reliable readout of ISV identity (vISV or aISV), based on the direction of flow (see Figure S2 ans supplementary S6 for adult vasculature). For ISVs without observable blood flow, *flt1*^{enh}*:RFP*^{high} ISVs were invariably connected to the dorsal aorta, whereas *flt1*^{enh}*:RFP*^{low} ISVs were connected to the PCV.

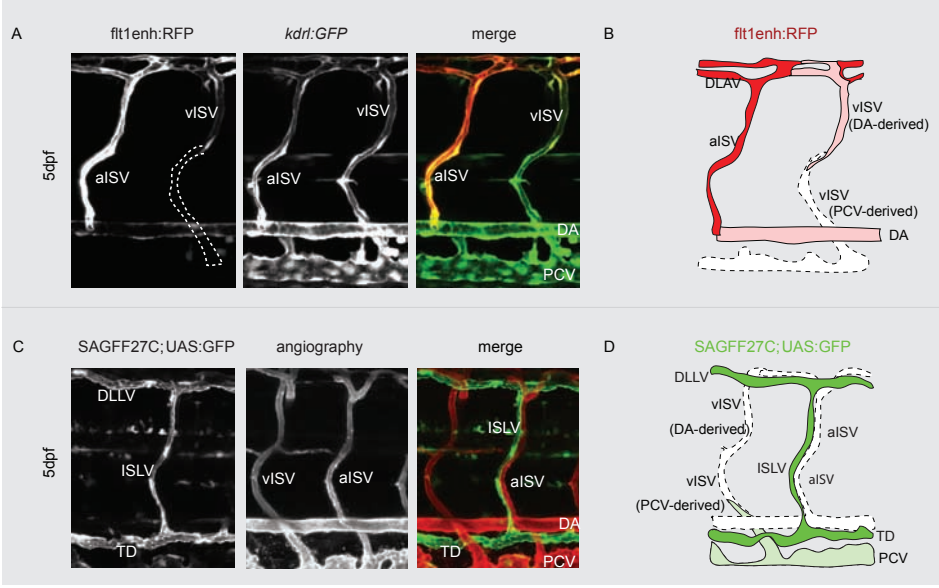


Figure 1: Generation and visualization of flt1enh:RFP and SAGFF27C;UAS:GFP transgenic lines. (A) Arterial-restricted reporter expression in the flt1enh:RFP transgenic line at 5 dpf ,compared with kdrl:GFP, which labels all blood endothelial cells allows for discrimination between ISVs connected to the dorsal aorta or PCV. (B) Schematic representation of flt1enh:RFP expression. (C) Lymphatic reporter expression in the SAGFF27C;UAS:GFP transgenic line at 5 dpf, compared with an angiography (F) that labels perfused blood vessels. (D) Schematic representation of SAGFF27C;UAS:GFP expression. aISV, arterial ISV; DA, dorsal aorta; DLLV, dorsal longitudinal lymphatic vessel; ISLV, intersegmental lymphatic vessel; PCV, posterior cardinal vein; TD, thoracic duct; vISV, venous ISV.

Another line we have previously generated, *stab1^{BAC}:YFP*, labels venous endothelial cells and lymphatic endothelial cells, including parachordal lymphangioblasts (PLs), that are derived from them (Hogan et al., 2009). In this study, by performing a large-scale gene-trap screen using the T2KSAGFF gene trap construct, we identified the *SAGFF27C* transgenic line that, when crossed to UAS:GFP fish, displayed reporter expression at much higher levels than *stab1^{BAC}:YFP*, facilitating high-resolution imaging. This line labels the lymphatic vasculature, i.e. the thoracic duct, DLLV and ISLVs (Figure 1C, D and Supplementary Figure S4, S5 for adult blood vasculature)

Arterio-venous and lymphatic patterning in the zebrafish trunk

Interestingly, when combining both transgenic lines we observed that in *flt1^{enh}:RFP; SAGFF27C;UAS:GFP* transgenic embryos, *SAGFF27C;UAS:GFP^{+}* ISLVs appeared to be almost exclusively located along *flt1^{enh}:RFP^{high}* aISVs and only very rarely along *flt1^{enh}:RFP^{low}* vISVs. To confirm this observation, we quantified lymphatic and blood vascular patterning. To this end, we performed a detailed analysis of lymphatic and blood vascular patterning at 5 dpf, based on the expression of *flt1^{enh}:RFP* and *SAGFF27C;UAS:GFP* (Figure 2A). For a total of 30 embryos, the arterial or venous identity of ISVs and the presence of ISLVs in all segments was monitored (Figure 2B).

We first analyzed the arterio-venous patterning of the trunk vascular network and confirmed the previous observation obtained from six embryos (Isogai et al., 2001) that the first segmental positions are almost invariable and bilaterally symmetrical. We found a vISV at segmental positions 1, 3 and 4, and an aISV at position 2. Between position 5 and 25, the arterial or venous identity becomes essentially random, with 48% (605/1260) aISVs and 52% (653/1260) vISVs (Figure 2B, C). It has previously been reported that ISVs in neighboring segments and at the left and right side of the embryo have a tendency to have different identity (Isogai et al., 2001). Of these relationships, we could only find support for a correlation between ISV identities in neighboring segments at the same side of the embryo. This correlation is strong, but not absolute (which would otherwise result in invariable aISV-vISV-aISV-vISV alternations): in 78% (928/1256) of ISVs between position 5 and 25, the ISV in the neighboring segment is of different identity. The other postulated relationship between vessel identities within the same somite pair we found to be uncorrelated (49% different identity (310/628). We additionally found a significantly higher likelihood for arterial identity in the caudal-most ISVs: 92% (55/60) of these are arterial (Figure 2D). As the number of segmental positions varies slightly (26 and 29) between embryos, this leads to a statistically significant enrichment of aISVs at, and posterior to, position 26 (Figure 2B, top panel).

Second, we analyzed the patterning of the lymphatic network. Interestingly, lymphatic ISVs were only found from segment 5, whereas the first four (invariably patterned) ISVs were never flanked by an ISLV. Posterior to this position, on average one ISLV (out of a potential two) was identified per somite pair within a segment. The percentage of ISLVs dropped from segmental position 18 (Figure 2B), indicating a rostral-to-caudal succession of lymphatic migration, similar to that observed for

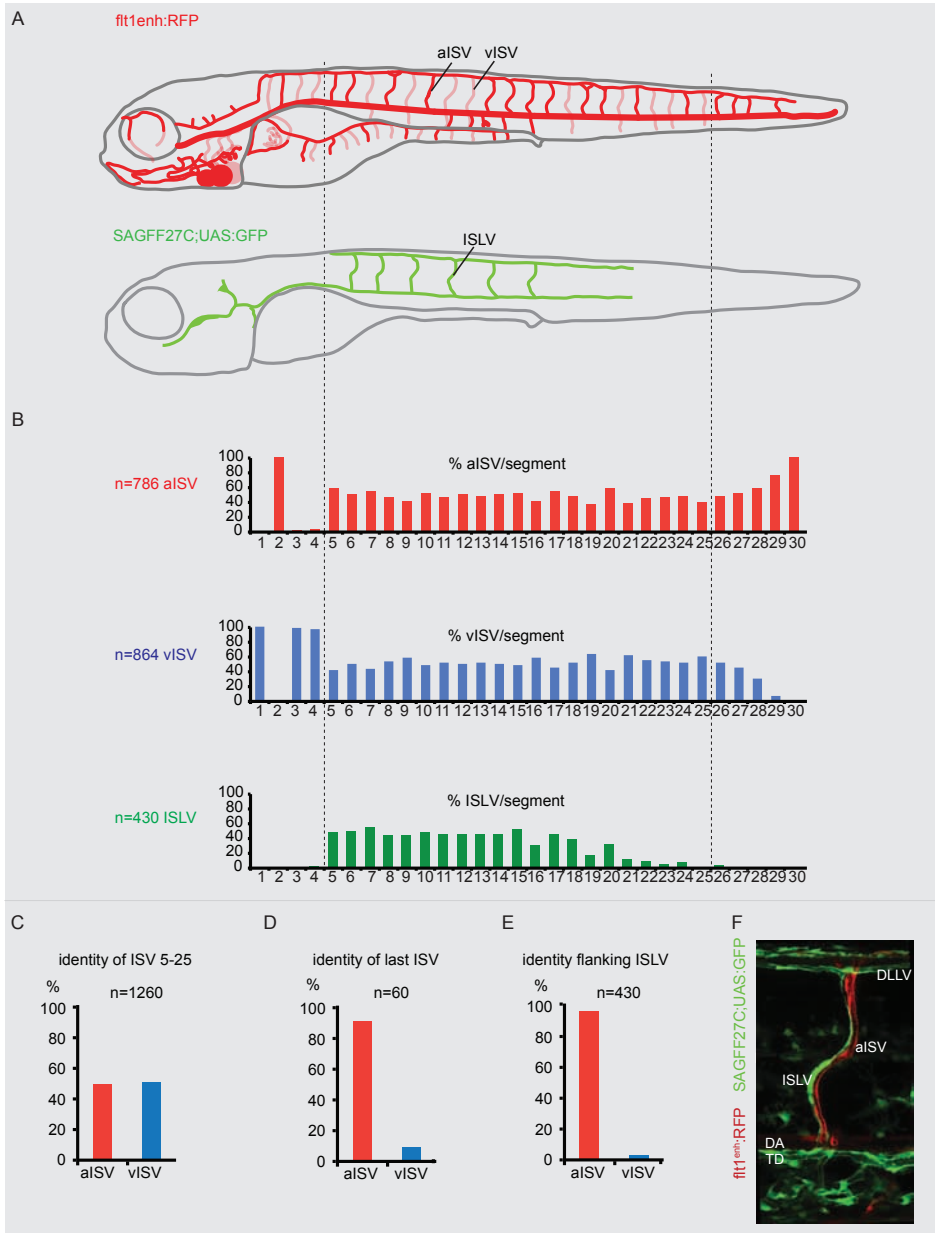


Figure 2: Arterio-venous and lymphatic patterning in the zebrafish trunk. (A) Schematic representation of arteries, veins and lymphatic vessels at 5 dpf, based on expression of flt1enh:RFP and SAGFF27C;UAS:GFP over 25-30 segments in 30 embryos (segment numbers vary in zebrafish embryos, with an average of 27.2 segments or 54.4 segmental positions per embryo). (B) Quantification of vessel type distribution per segment, indicating aISV in red bars, vISVs in blue bars and ISLVs in green bars. (C) Quantification of aISV/vISV identity in segments 5-25, n=1260 segmental positions scored. (D) Quantification of aISV/vISV identity of the caudal-most ISV/side, n=60 indicating a strong preference for aISV over vISV in the last ISV position. (E) Quantification of arterial and venous ISVs abutting an ISLV, n=430 intersegmental vessel pairs scored. (F) Overlaid confocal images of flt1enh:RFP+ aISV and SAGFF27C;UAS:GFP+ ISLV. aISV, arterial ISV; DA, dorsal aorta; DLLV, dorsal longitudinal lymphatic vessel; ISLV, intersegmental lymphatic vessel; TD, thoracic duct; vISV, venous ISV.

primary angiogenic sprouting. Consistently, at 7 dpf, ISLVs were observed at more caudal positions (data not shown). Even though PLs have a choice of either vISVs and aISVs at the time when they migrate dorsally, 97% (418/430) of ISLVs were found neighboring aISVs, suggesting an important role for arteries in regulating lymphatic patterning (Figure 2E, F).

Intersegmental arteries are required for LEC migration

The remarkable alignment of ISLVs with arteries/aISVs suggests that aISVs act as guides for lymphatic patterning. Alternatively, lymphatic precursors could initially show no preference for arteries over veins, but become attracted to aISVs later. To distinguish between these two possibilities, we performed time-lapse confocal imaging in the *flt1*^{enh}*:RFP; SAGFF27C;UAS:GFP* transgenic line. We found that PLs migrate away from the horizontal myoseptum (and are now referred to as LECs) almost exclusively along aISVs, both ventrally to form the thoracic duct, as well as dorsally to form the ISLV and DLLV (Figure 3A; see Movie 1 in the supplementary material). At the stages of development we analyzed, no vascular smooth muscle cells or pericytes have been identified in the intersegmental network of the zebrafish. In addition, we show that migrating LECs use aISVs as a direct substrate for migration: PLs (and LECs) extend protrusions along the external surface of aISVs (Figure 3B, C; see Movies 2, 3 in the supplementary material) before they migrate along the surface to extend ventrally in the direction of the DA and dorsally in the direction of the DLAV. These findings strongly suggest that the endothelial cells from arterial ISVs are regulators of LEC migration in the zebrafish.

These observations do not rule out the possibility that arteries are simply a preferred substrate for lymphatic precursor migration, and that in the absence of arteries, lymphatic endothelial cells might be able to migrate completely independently or on vISVs. To investigate this possibility, we made use of *plcy*^{t26480} and *kdrl*^{hu5088} mutants that displayed reduced or no primary (arterial) angiogenic sprouting, but retained normal secondary (venous) angiogenic sprouting and PL formation. In *plcy*^{t2648} mutants, we did not observe ISLV or TD formation (data not shown). However, as *plcy*^{t26480} mutants do not establish trunk circulation and display signs of necrosis at the time of our analysis (3-5 dpf), we performed the analysis in zebrafish that are mutant for the vascular endothelial growth factor receptor *kdr-like* (*kdrl*^{hu5088}). These mutants retain circulation in the DA and PCV, and form LECs and a TD. Mutants and morphants have some residual primary angiogenic sprouts. These sprouts were usually found only ventral to the horizontal myoseptum, therefore no DLAV was established. Venous sprouts were occasionally found to connect to these small vessels, establishing a short functional circulatory loop. In *kdrl*^{hu5088} mutants, PLs were found to migrate away from the horizontal myoseptum, but only ventrally along the residual aISVs, but not along vISVs. This led to the establishment of a thoracic duct at the ventral side, but not to the formation of a DLLV at the dorsal side of the embryo (Figure 4A).

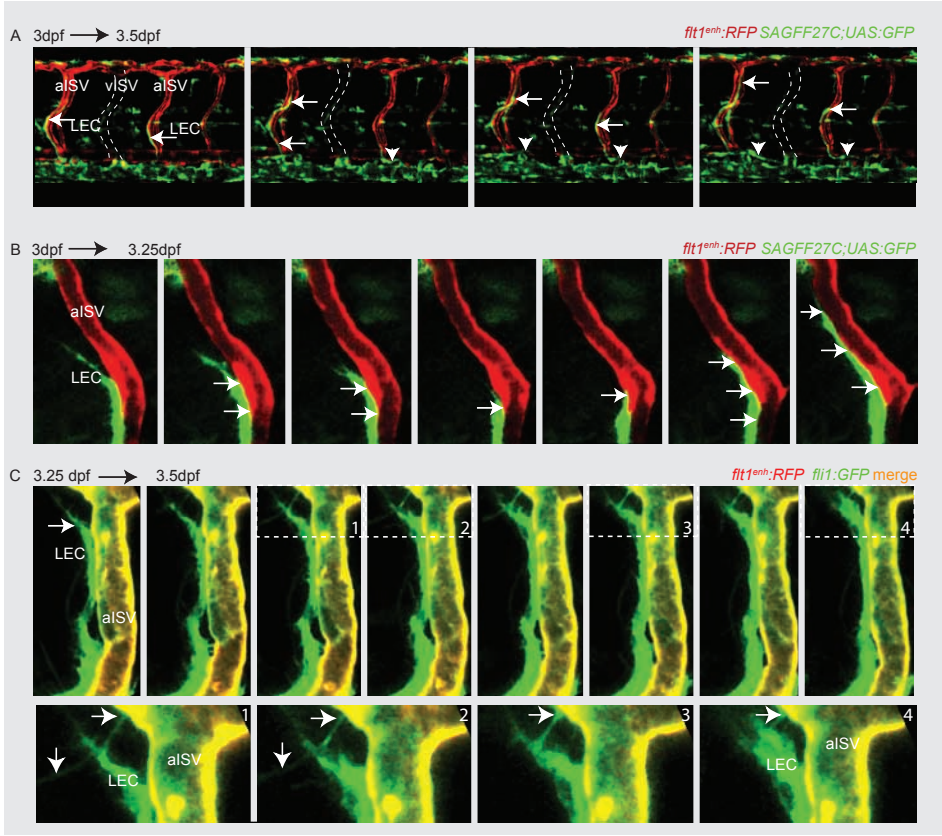


Figure 3: Migration of lymphatic precursors along intersegmental arteries. (A) Still images of flt1enh:RFP;SAGFF27C;UAS:GFP triple transgenic embryos reveal migration of SAGFF27C;UAS:GFP+ LECs (arrows) along flt1enh:RFP+ arteries and establishing the TD (arrowheads) in the trunk. The position of a vISV is indicated by broken lines. (B) Still images of migrating SAGFF27C;UAS:GFP+ LECs suggest direct contact (arrows) between the aISV and the LEC. (C) Still images of migrating fli1a:GFP+ LECs and filopodia formation (indicated by arrows) at the leading edge along the surface of a flt1enh:RFP+; fli1a:GFP+ artery at 3 dpf. Areas above the broken lines are shown at higher magnification in the bottom row.

This result shows a requirement for aISVs in lymphatic precursor migration. Occasionally, aISVs were formed that extended to the dorsal side of the embryo. When this occurred, lymphatic precursors were also able to migrate dorsally (see Figure S3 in the supplementary material), thereby demonstrating that arteries are crucial for proper guidance of LEC migration, and that *kdr-like* does not play a role in the process.

Our data provide the first in vivo demonstration of lymphatic endothelial cells to be dependent on arterial ECs for pathfinding. Time-lapse analysis strongly suggests a physical interaction of these types of endothelial cells. This interaction does not depend on the *kdr-like* VEGF receptor, as in those few cases where arterial ISV were observed in mutants (see Figure S3 in the supplementary material), PLs did migrate

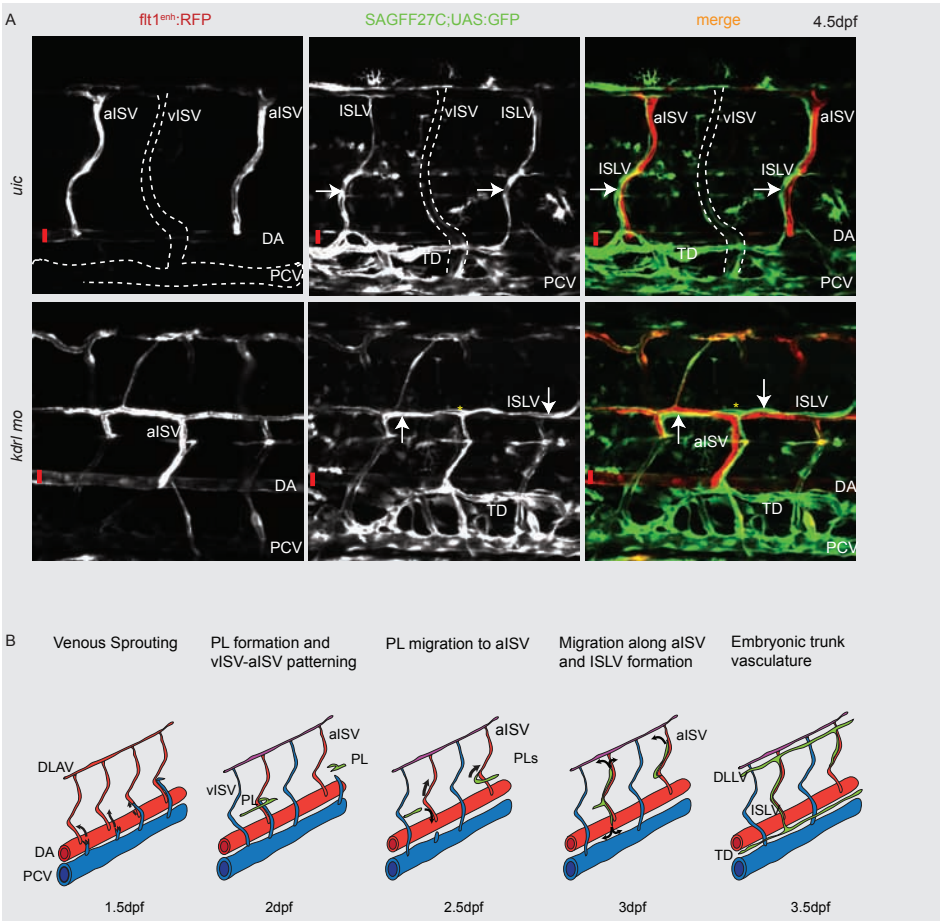


Figure 4: Intersegmental arteries are required for LEC migration. (A, top panels) flt1enh:RFP, SAGFF27C;UAS:GFP and merged unilateral confocal images of control embryos at 4.5 dpf showing lymphatic patterning along aISVs in normal blood vasculature. Arrows indicate ISLVs. (A lower panels) flt1enh:RFP, SAGFF27C;UAS:GFP and merged unilateral confocal images of kdrl morpholino injected embryos at 4.5 dpf showing LEC migration along arteries that cross-connect abnormally at the horizontal myoseptum, and that do not extend into the dorsal half of the respective somite. Arrows indicate LECs along the surface of an abnormal aISV. Vertical red bars indicate the DA. (B) Model of lymphatic patterning within the zebrafish trunk. Secondary sprouts from the PCV that do not connect to primary intersegmental vessels migrate to the horizontal myoseptum region where they constitute a pool of PLs. PLs will migrate ventrally or dorsally to form the thoracic duct and ISLVs, respectively, and use arteries as their substrate.

along arteries. This would predict that deleting arteries by other means would also interfere with this process. Indeed, suppressing the Notch pathway by DAPT treatment of embryos, which results in a shift from arterial to venous fates (Lawson et al., 2001) effectively blocks migration of PLs dorsally (Geudens et al., 2010) consistent with our model and the situation in *kdr-like* mutants and morphants (Figure 4B).

The possible contact-dependent migration of LECs on arterial ECs provides a new paradigm for LEC pathfinding. Several possibilities could be explored to unravel more insight into the mechanism regulating LEC migration. The secretion of guidance factors for LECs by arterial ECs, such as VEGF-C, is one possibility, a shared preference of both PLs/LECs and aISVs for the same molecular environment or the same guidance cues (such as semaphorins, integrins or other extracellular matrix molecules) is another. Furthermore, arterial ECs might deposit ECM components that provide crucial substrate components for LECs. Further studies will have to unravel which molecules are central to the interaction between these two types of endothelial cells.

MATERIAL AND METHODS

Zebrafish strains and morpholino injections

All zebrafish strains were maintained under standard husbandry conditions. The *kdrl*^{hu5088} allele was identified in a forward genetic screen (Hogan et al., 2009) *Tg(fli1a:gfp)*^{y1} (Lawson and Weinstein, 2002b) and a *kdrl* morpholino (Habeck et al., 2002) have been described previously.

Transgenesis

flt1 promoter/enhancer constructs were cloned in the Mini*Tol2* vector (Balciunas et al., 2006) and injected at 25 ng/µl with tol2 transposase mRNA (25 ng/µl) into eggs. Embryos were selected at 3 dpf for high expression and grown to adults, among which germline founders were identified.

The SAGFF27 line was identified in a large scale gene-trap screen (Asakawa et al., 2008) using the T2KSAGFF construct. The insertion in this line occurred in the first intron of zgc:171516 (ZFIN) and is located on chromosome 24. Characterization of the homozygous phenotype will be described elsewhere (A.U. and K.K., unpublished). Heterozygous fish were viable and fertile, and did not display obvious defects in blood or lymphatic vascular patterning.

Imaging

Microangiography was performed as described (Kuchler et al., 2006)Embryos were mounted in 0.5-1.0% low melting point agarose (Invitrogen, Carlsbad, CA, USA) in E3 buffer. Confocal image stacks were collected on SPE, SP2 or SP5 confocal microscopes with 10×, 20×, 40× and 63× objectives (Leica Microsystems, Wetzlar, Germany) and processed using ImageJ (http://rsbweb.nih.gov/ij)

ACKNOWLEDGEMENTS

S.S.-M. was supported by the KNAW and J.B. by the Vrienden van het Hubrecht Stichting. F.L.B. was supported by a grant from NWO Medical Sciences (Vidi) to H.J.D. The SP5 microscope of the Hubrecht Imaging Centre was supported by NWO grant 175.010.2007.007. We thank the Hubrecht Imaging Centre for support. The new *kdrl*^{hu5088} allele was uncovered by B. Hogan. C. Veelken helped in the molecular analysis. We thank the National BioResource Project of Japan and acknowledge grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan. We also want to thank all members of the Schulte Merker Lab for discussions and T. Karpanen for comments on the manuscript.

SUPPLEMENTAL DATA

Online available at: http://dev.biologists.org/content/137/16/2653/suppl/DC1

SUPPLEMENTAL FIGURES

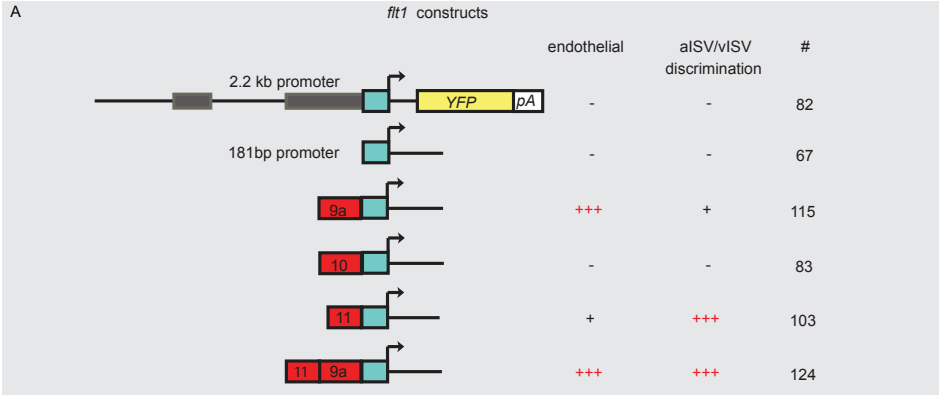


Figure S1: Identification and generation of *flt1*^{enh}*:RFP* locus. Identification of conserved non-coding elements (CNEs) within the zebrafish *flt1* locus. CNEs included on BAC DKEY 256I8 (which was used to generate the *flt1*^{BAC}*:YFP* transgenic line)(Hogan et al., 2009) were identified by 11-way Mulan alignment (Ovcharenko et al., 2005). Conserved coding elements are in blue, conserved non-coding elements (CNEs) are pink (intronic), yellow (3'UTR) or red (intergenic). 12 CNEs were identified. Of these, 3 were conserved in all vertebrates analyzed (CNE 9a, 10 and 11, indicated in red) and tested for enhancer activity. A. Overview of tested *flt1* enhancer constructs, and results from transient injections indicating presence of endothelial expression and degree of arterial versus venous discrimination among ISVs. Grey boxes in the 2.2kb promoter construct indicate repetitive elements. #: number of embryos analyzed. *Flt1* promoter-YFP-polyA fragments (2.2kb and 181bp) were amplified from the modified BAC DKEY-256I8 used for the generation of the *flt1*^{BAC}*:YFP* transgenic line (Hogan et al., 2009) and cloned into the pMiniTol2 (Balciunas et al., 2006) vector to generate pTol2_flt1p2.2kb_YFP and pTol2_flt1p181_YFP. Individual enhancer elements were cloned into pTol2_flt1p181_YFP.

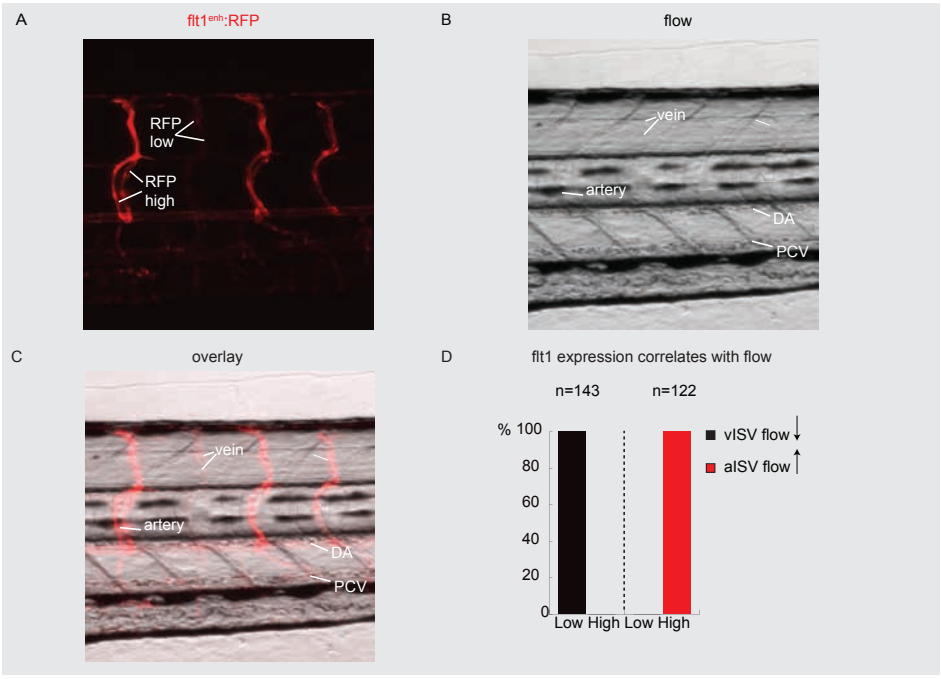


Figure S2: *flt1^{enh}:RFP* expression correlates with flow. Comparison between *flt1^{enh}:RFP* expression and direction of blood flow in the trunk intersegmental network. A, *flt1^{enh}:RFP* expression at 5 dpf reveals two types of ISVs with either high or low RFP expression. B. Still frame of direction of flow in a 5 dpf *flt1^{enh}:RFP* embryo revealing arteries (up from DA) and veins (down to PCV) C. Overlay of *flt1^{enh}:RFP* and flow reveals that arteries express *flt1^{enh}:RFP* at high and veins express *flt1^{enh}:RFP* at low levels. B, Quantification of flow direction and expression of flt1^{enh}:RFP. n=143 aISV and n=122 vISV.

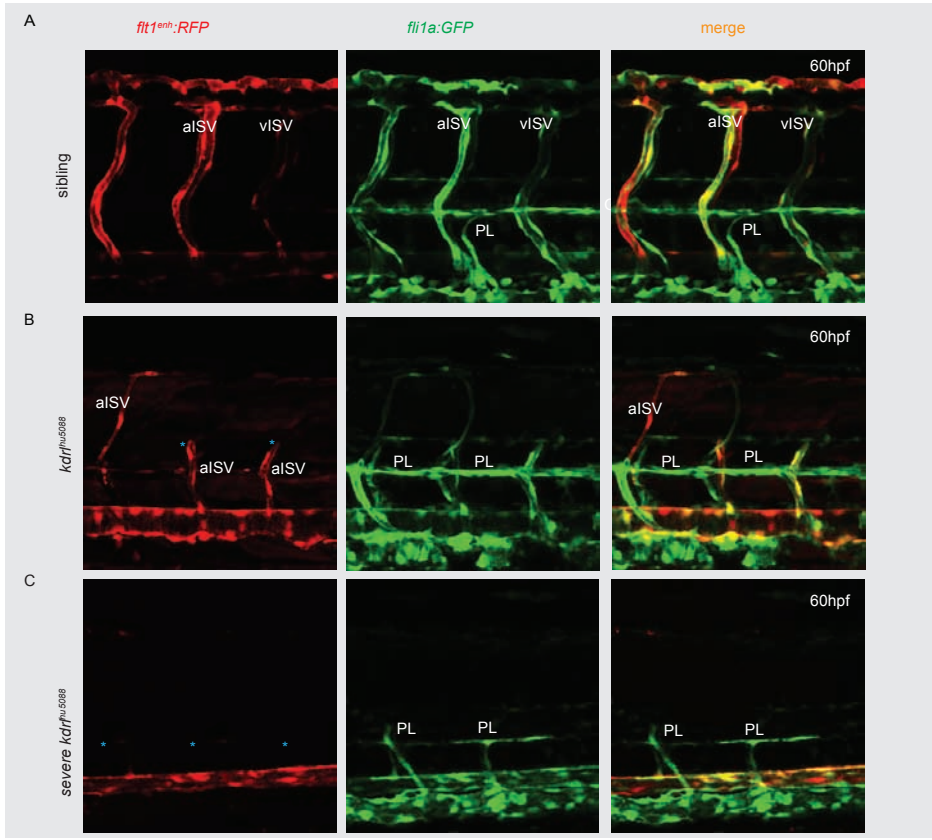
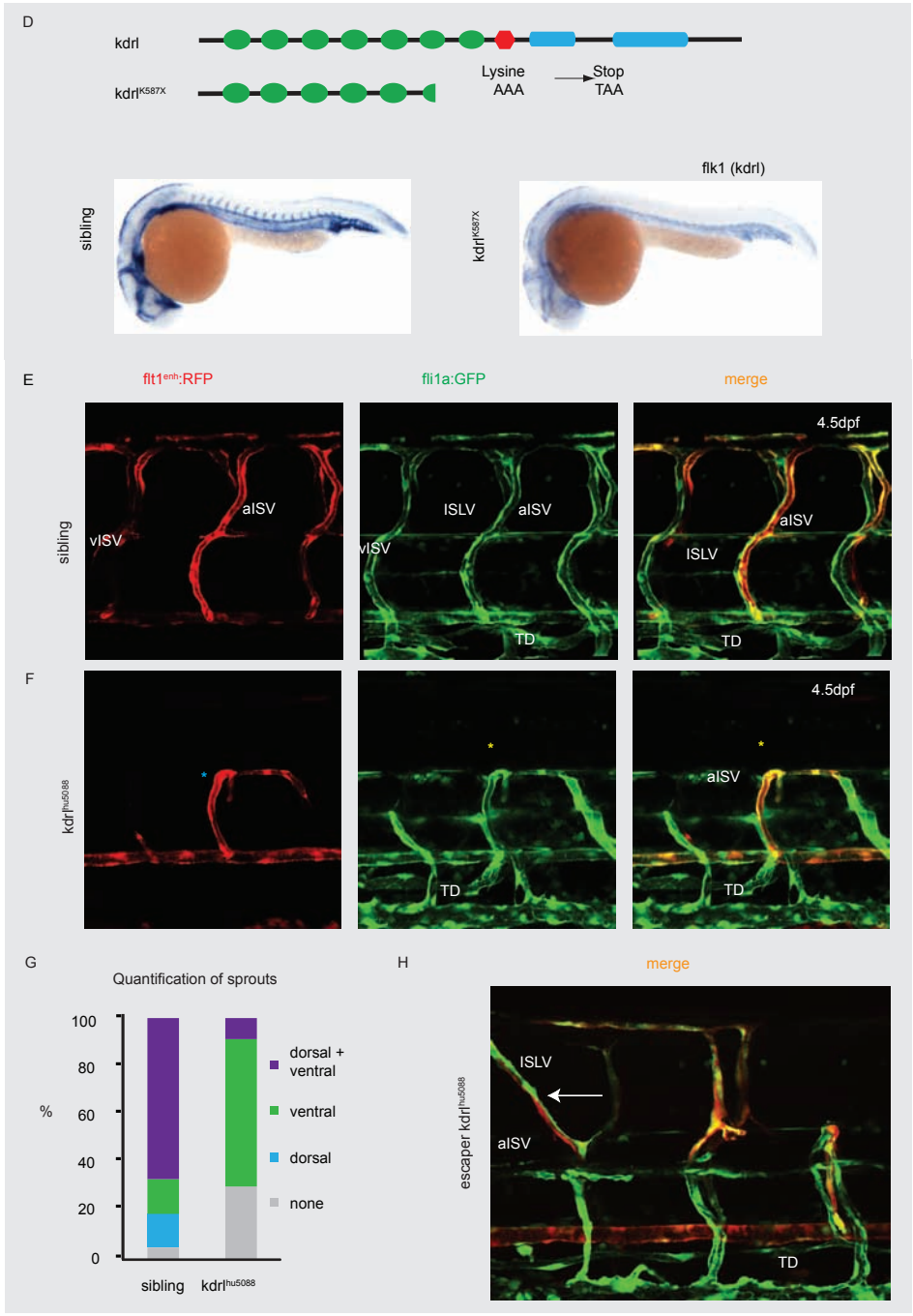


Figure S3. Analysis of angiogenesis mutant *kdrl*^{hu5088}. (A-C) 60 hpf sibling and mutant *kdrl*^{hu5088} in *fli1a:GFP; flt1*^{enh}*:RFP* double transgenic embryos reveal that PL formation is normal, whereas the primary sprouts can be affected differently (indicated by the yellow asterisk) in mutants. (D) *Kdrl*^{hu5088} failed to complement *kdrl*^{t21588} (Habeck et al., 2002) and sequencing revealed an A-to-T transversion, changing a lysine at amino acid position 587 into a premature stop (K587X) resulting in reduced *kdrl* expression. (E-F) Lymphatic vasculature establishment in 4.5 dpf sibling and mutant *kdrl*^{hu5088} *fli1:GFP; flt1*^{enh}*:RFP* double transgenic embryos with normal or with reduced primary sprouts at 4.5 dpf. Mutants have reduced primary sprouting (indicated by blue asterisks) and lack dorsal migration of LECs. (indicated by yellow asterisks), whereas ventral migration is still present. G Quantification of dorsal and ventral migration along arteries. Mutants with thoracic duct (n=5, total of 23 arteries) without dorsal migration, whereas the ventral migration along these arteries is mostly retained. H. *kdrl*^{hu5088} 'escapers' in *fli1:GFP*, *flt1*^{enh}*:RFP* double transgenic embryos at 4.5 dpf. LECs (arrows) migrate along escaping arteries that did succeed in growing dorsally.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Quantification of sprouts
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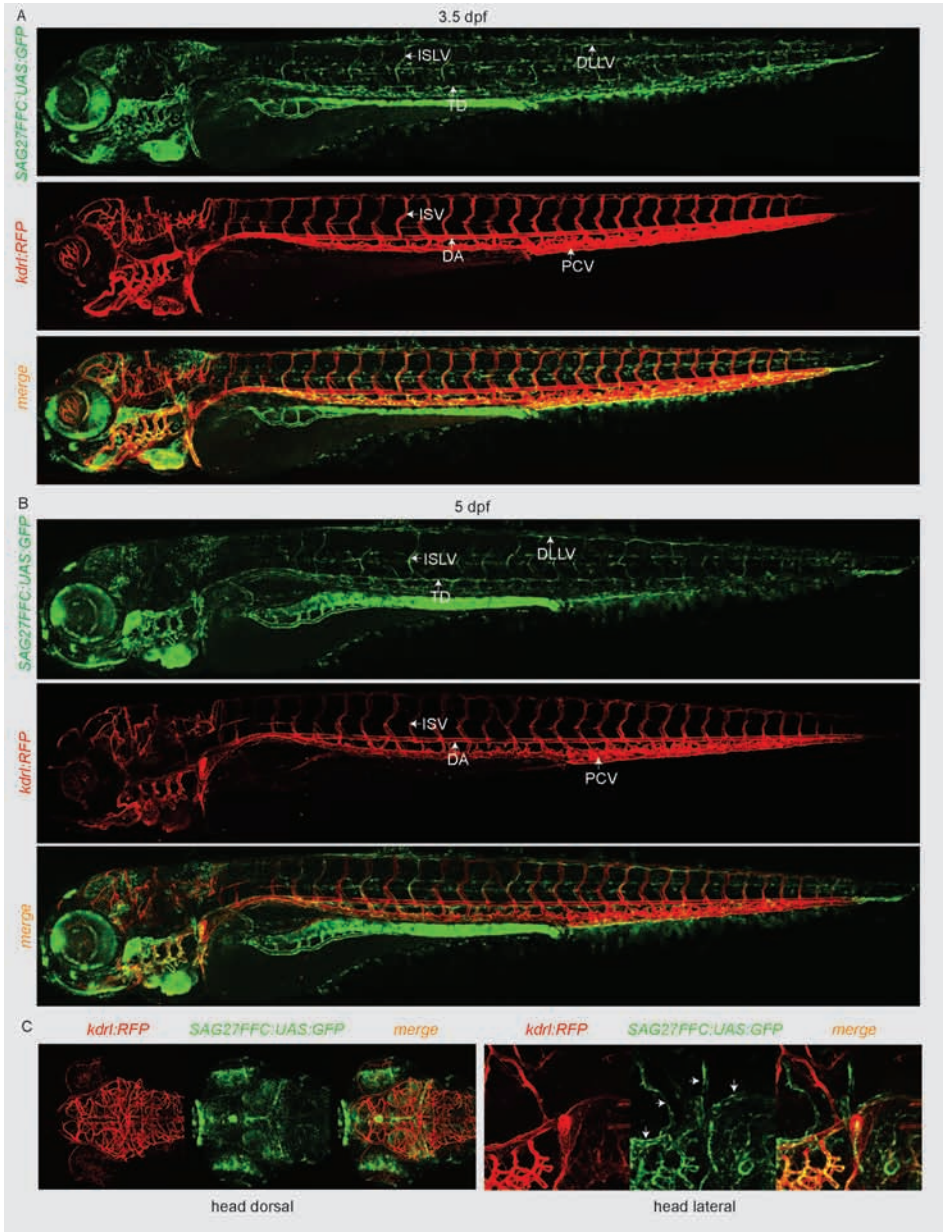


Figure S4: Embryonic lymphatic vasculature of kdrl:RFP and SAGFF27C;UAS:GFP transgenic lines. (A) Endothelial kdrl:mcherry (red) and SAGFF27C;UAS:GFP (green) lymphatic reporter expression at 3.5 and (B) 5 dpf which labels all blood endothelial cells (red) and lymphatics (green). (C) Head (dorsal and lateral) vasculature of endothelial kdrl:GFP and SAGFF27C;UAS:GFP lymphatic reporter expression showing lymphatic structures (indicated by arrows).

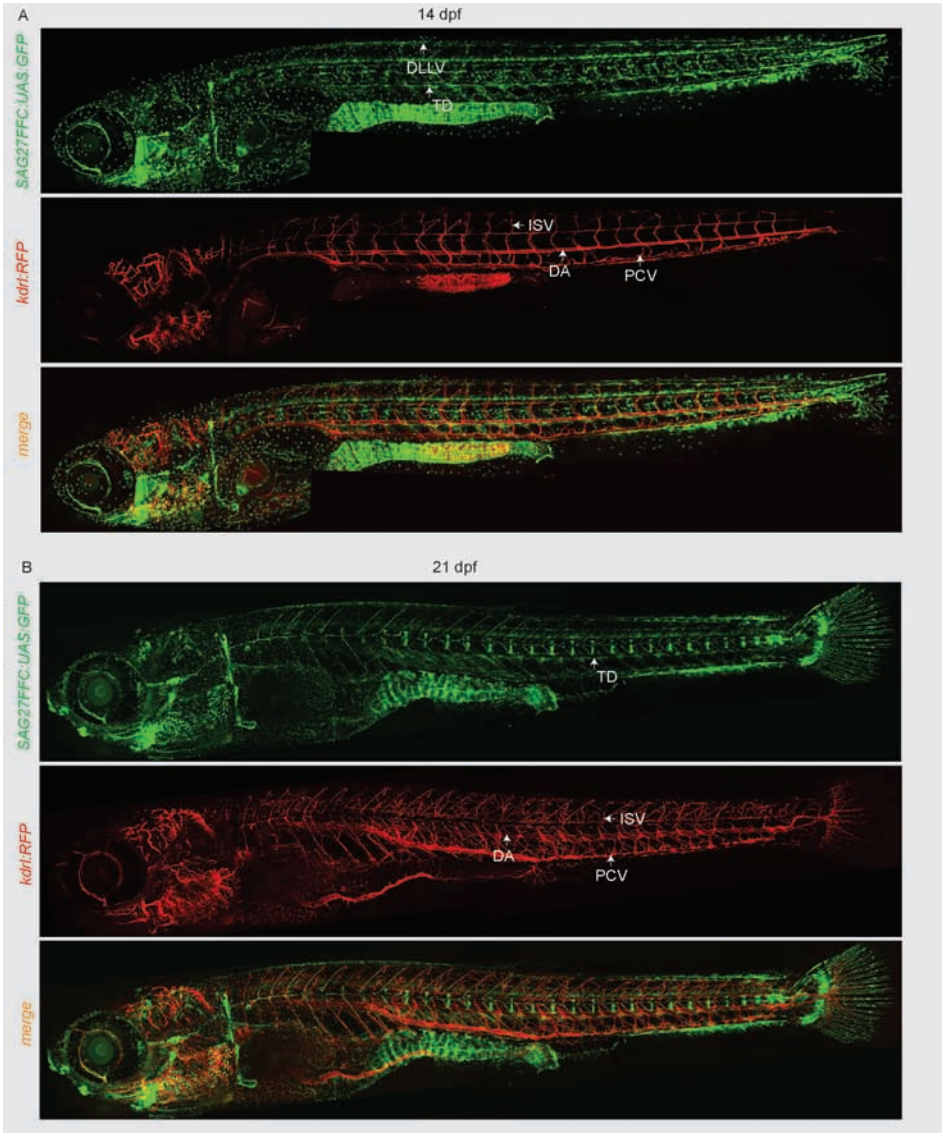


Figure S5: Adult vasculature of kdrl:mcherry and SAGFF27C;UAS:GFP transgenic lines. (A) Endothelial kdrl:mcherry (red) and SAGFF27C;UAS:GFP (green) lymphatic reporter expression at 14dpf and (B) 21 dpf which labels all blood endothelial cells and lymphatics. The expression of SAGFF27C;UAS:GFP is also detected in bone elements, and becomes less restricted to the lymphatic vasculature over time.

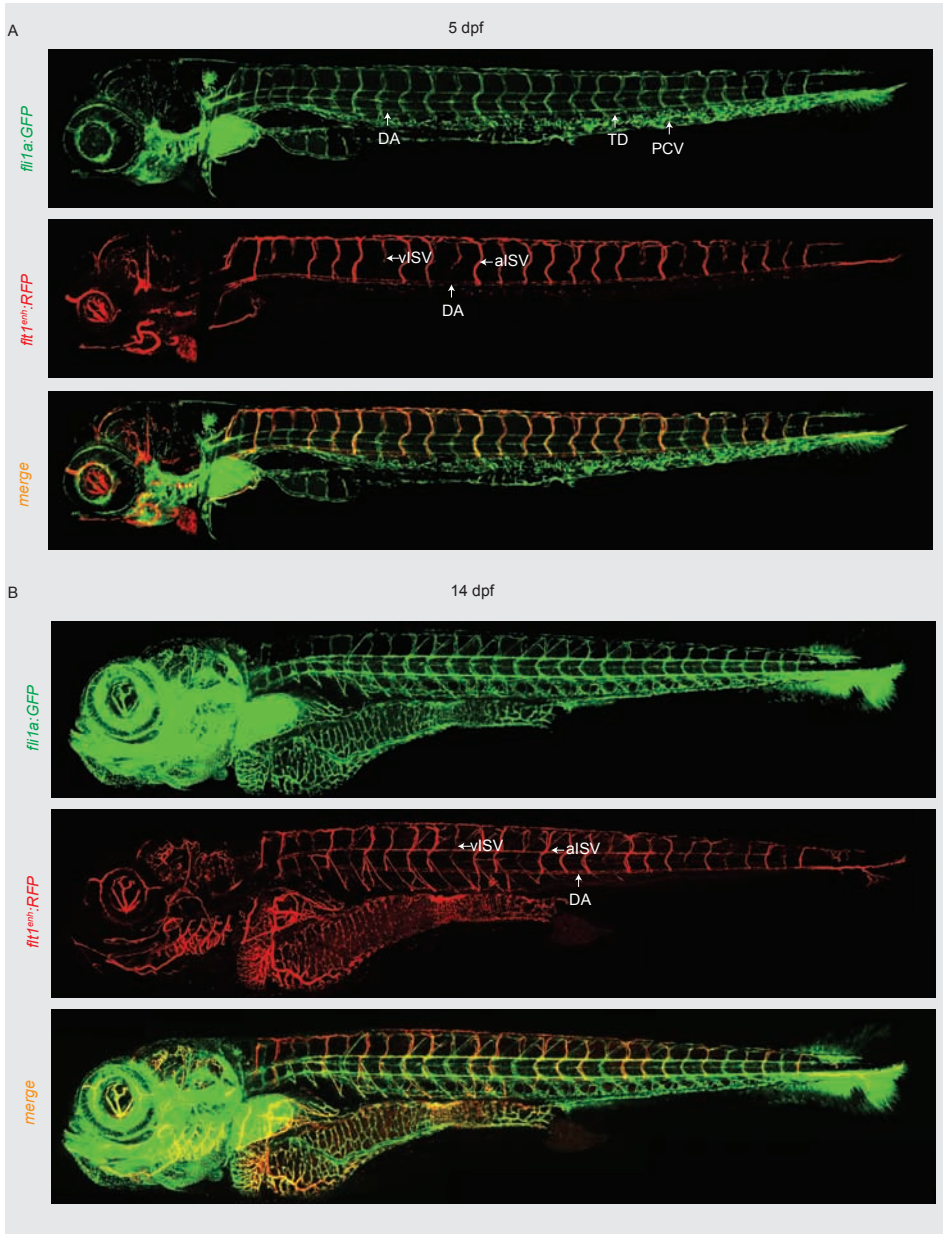
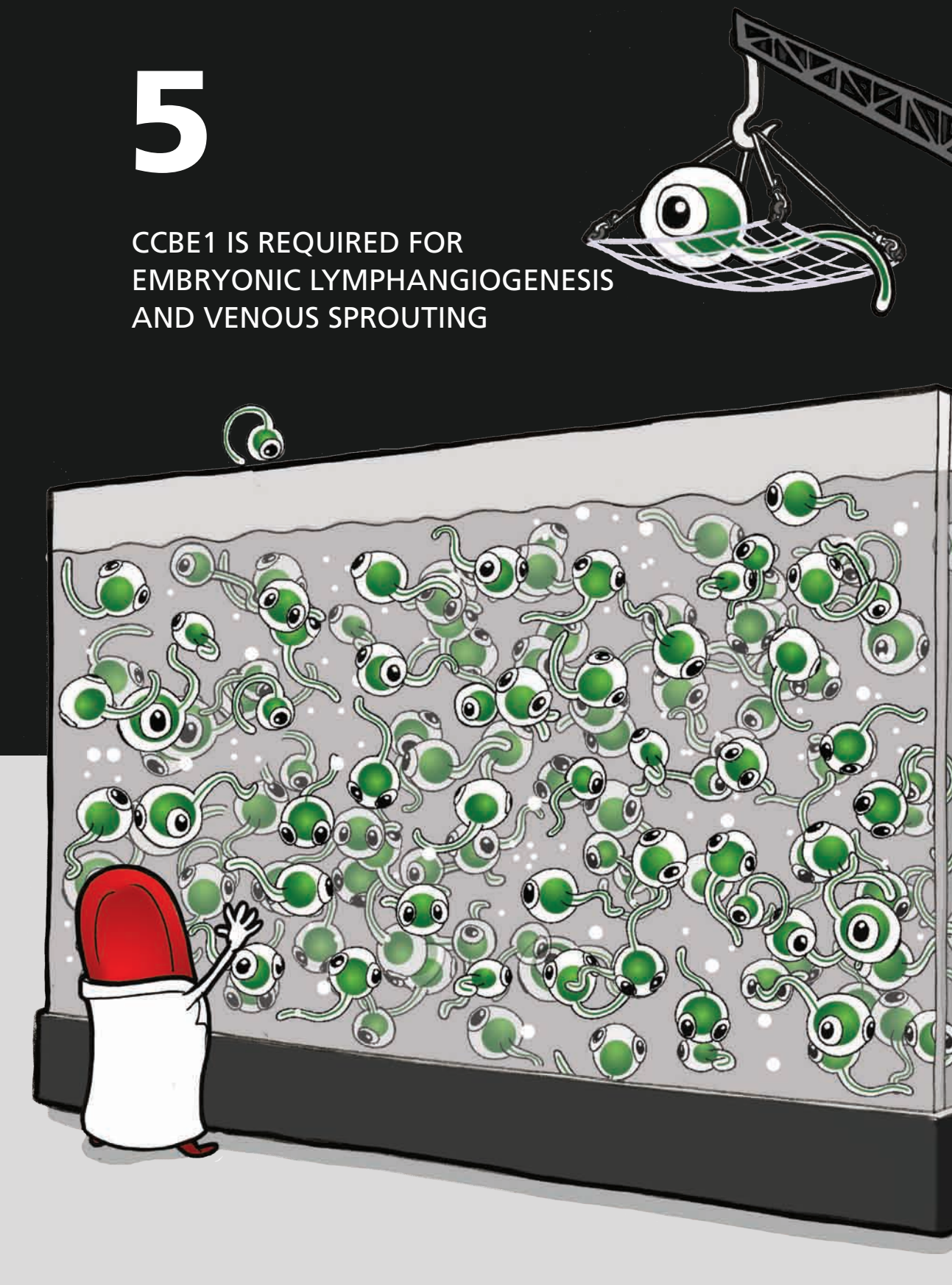


Figure S6: Embryonic and adult arterial vasculature of fli1a:GFP and flt1enh:RFP transgenic lines. (A) Endothelial fli1a:GFP (green) and flt1enh:RFP (red) arterial reporter expression at 5 dpf, 14 dpf (B) and 21 dpf (C) which labels and discriminates arterial endothelium from all endothelium.

5

CCBE1 IS REQUIRED FOR EMBRYONIC LYMPHANGIOGENESIS AND VENOUS SPROUTING



Benjamin M. Hogan [1], **Frank L. Bos** [1,2] *, Jeroen Bussmann [1] *,
Merlijn Witte [1] *, Neil C. Chi [4], Henricus J. Duckers [2]
& Stefan Schulte-Merker [1]

1. Hubrecht Institute-KNAW & University Medical Centre, Utrecht, The Netherlands;
2. Experimental Cardiology, Thoraxcenter, Erasmus University Medical Center, Rotterdam, The Netherlands;
3. Department of Medicine, University of California San Diego, Division of Cardiology, La Jolla, California, USA.

* Authors contributed equally

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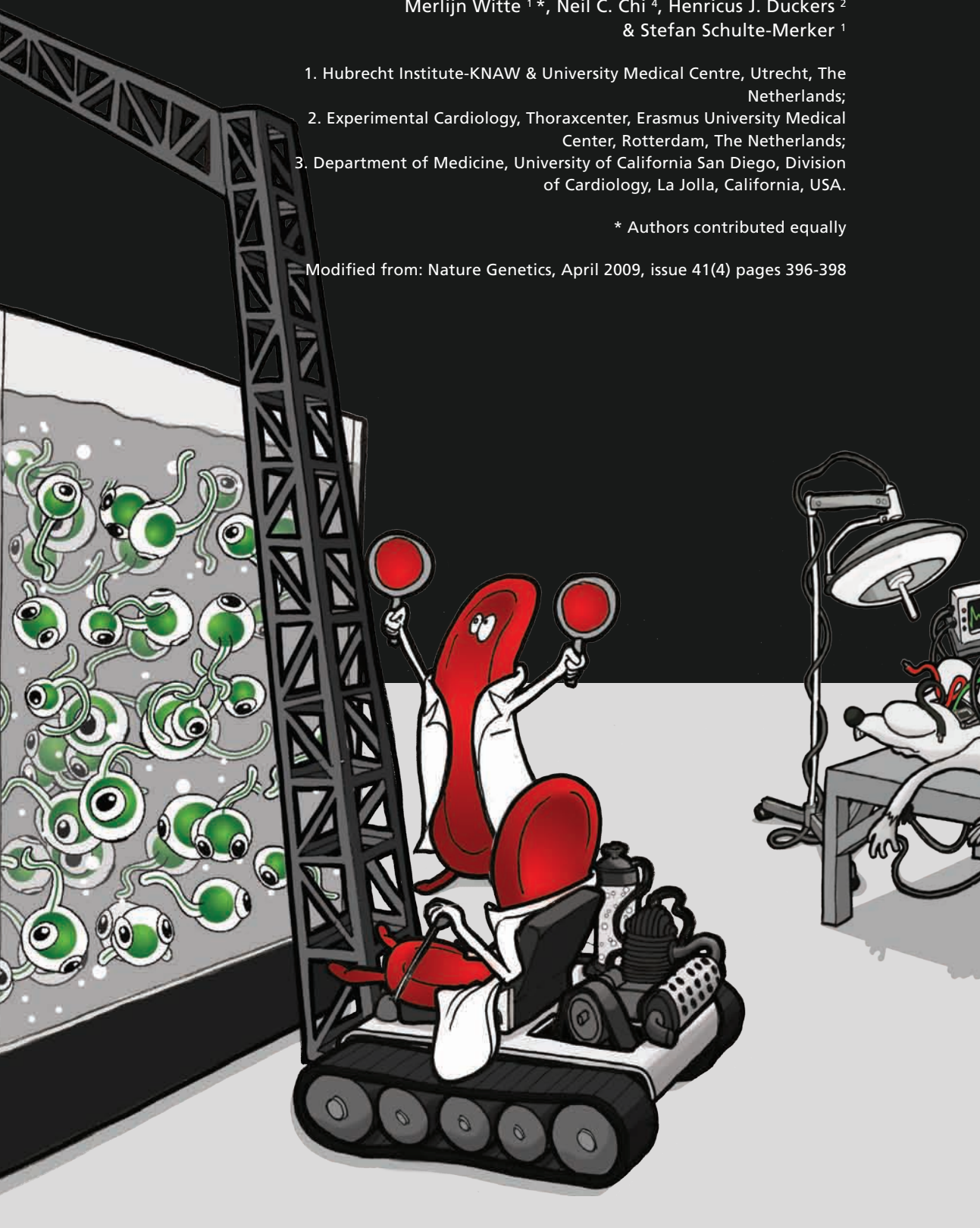

SUMMARY

Lymphatic vessels have important roles in fluid homeostasis, fat absorption, inflammation and cancer metastasis and develop in a dynamic process (called lymphangiogenesis) involving budding, migration and proliferation of lymphangioblasts. Using a genetic screen in zebrafish we identify *ccbe1* (*collagen and calcium-binding EGF domain-1*) as indispensible for embryonic lymphangiogenesis. Ccbe1 acts at the same stage of development as Vegfc and is required for lymphangioblast budding and angiogenic sprouting from venous endothelium.

RESULTS AND DISCUSSION

To identify regulators of lymphangiogenesis, we used the recently characterized zebrafish lymphatic vasculature (Kuchler et al., 2006; Yaniv et al., 2006) as a model. In a forward genetic screen we isolated one mutant, *full of fluid* (*fof*^{hu3613}), which lacked the thoracic duct and the previously unidentified intersegmental (ISLVs) and dorsal longitudinal lymphatic vessels (DLLVs) but retained a seemingly normal blood vasculature (Figure 1A-F). Mutants showed edema, initiating in the lower intestine and around the eyes from 6 d post fertilization (dpf) (Figure 1 G,H). At 36 dpf, surviving mutants showed severe edema (Figure 1I, J)

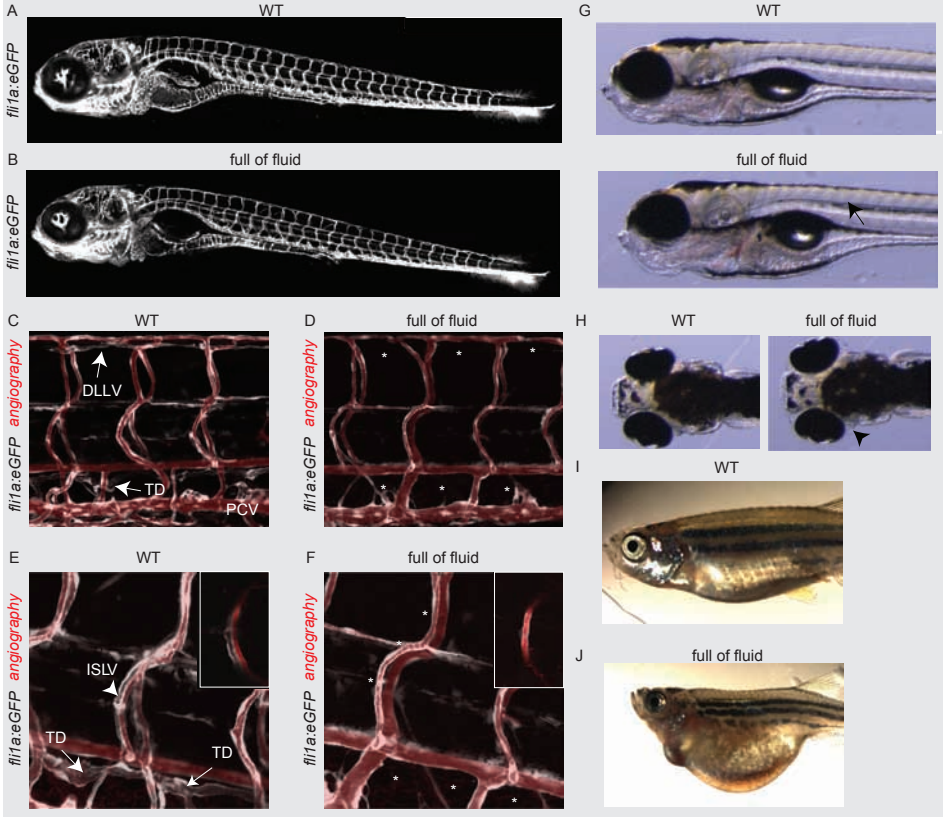

Figure 1: *full of fluid* mutants lack trunk lymphatic vessels. A, B. Overall patterning of blood vessels (*fli1:GFP*, 5 dpf) in wild-type siblings (A) and *fof* mutants (B). C-F. Angiographies in *fli1:GFP* embryos at 5 dpf reveal that thoracic duct (arrows in C and E), intersegmental lymphatic vessels (ISLVs) (arrowheads and inset in E) and dorsal longitudinal lymphatic vessel (DLLV) (arrow in C) are devoid of blood flow. *fof* mutants lack all trunk lymphatic vessels (D and F). G, H. fluid accumulation in intestine(arrow in G) and around the eyes (arrowheads in H) in mutants (lower) compared with wildtype siblings (upper) at 6dpf. I,J. *fof* mutants (J) that survive to 36 dpf (n = 3/28, see Supplementary Figure 1) display severe edema.

Genetic mapping localized the mutation to chromosome 21 in an interval containing a single gene, *ccbe1* (*collagen and calcium binding EGF domain 1*) (Figure 2A). *ccbe1* encodes a predicted secreted protein containing a signal peptide, a collagen domain and a calcium-binding EGF domain. Sequencing revealed a coding mutation in the fourth exon of *ccbe1* changing a conserved aspartic acid (Asp162) residue to a glutamic acid in the calcium-binding EGF domain (Figure 2B-D). Notably, an equivalent substitution (D1479E) in a calcium-binding EGF domain of Fibrilin1 is associated with loss-of-function phenotypes in humans (http://www.umd.be/FBN1/). Injection of *ccbe1*-targeting morpholinos efficiently phenocopied *fof* at 5 dpf (Figure 2E-F) demonstrating that *fof*^{hu3613} is a loss-of-function allele. Wild-type but not mutant *ccbe1*^{D162E} mRNA injection rescued the *fof* phenotype without inducing excess vascular development (Figure 2G).

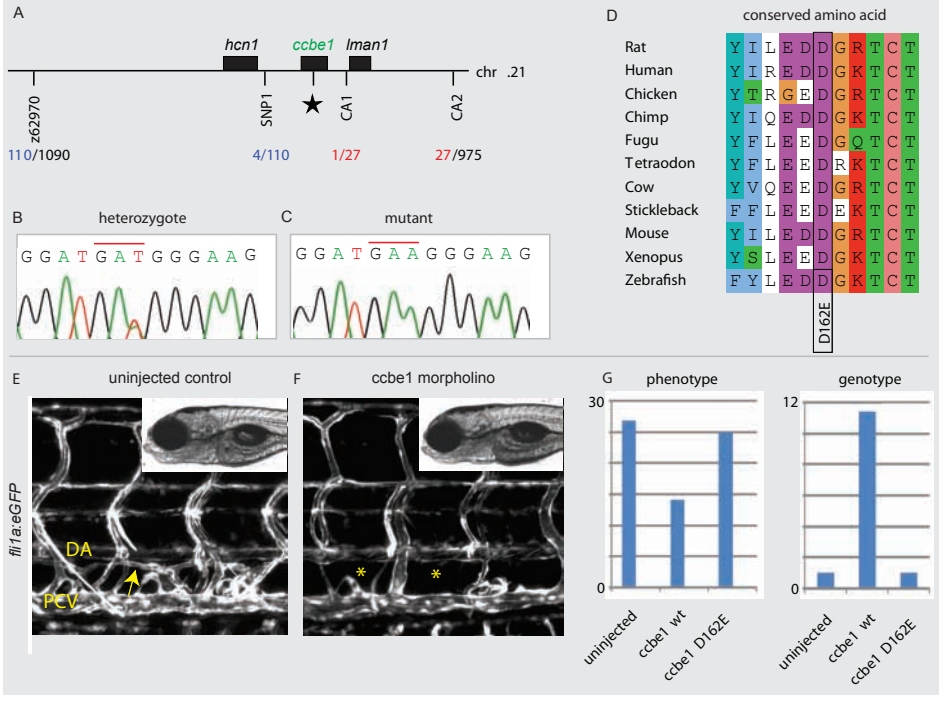

Figure 2: *fof*^{hu30613} encodes ccbe1. A. Meiotic map of the *fof* locus. Recombinant events for each polymorphic marker are depicted in red (proximal) and bleu (distal). Recombinants for SNP1 and CA1 exclude neighboring genes *lman1* and *hcn1*. B ,C. Sequencing of *ccbe1* identified a tranversion (T to A) corresponding to position 162 of the predicted protein, replacing aspartic acid (D) with glutamic acid (E) (affected codon underlined) d. Multiple sequence alignment of *ccbe1* proteins demonstrating the conservation of zebrafish D162E. E.F. Injection of ccbe1 ATG morpholinos phenocopies *fof* (F); wildtype control in (E). Arrow= thoracic duct, asterisk = absence of thoracic duct. G. *Ccbe1* mRNA rescued the *fof* phenotype. An incross of heterozygote adult *fof* carriers was injected with *ccbe1* mRNA (wt or mutant) and scored for the absence of the TD (phenotype). Subsequently, the embryo with a TD were genotyped and homozygous *fof* mutants were scored (genotype).

We observed restricted expression of *ccbe1* during development (Supplementary Figure 3 online) with expression in the pronephros and ventral mesenchyme at 24 h post fertilization (hpf) (Figure 3A). By 36 hpf, expression was detected in discrete zones along each somitic boundary, between the PCV and the horizontal myoseptum, as well as along the horizontal myoseptum itself (Figure 3A). At 48 hpf, *ccbe1* expression was restricted along the horizontal myoseptum (Figure 3B-C). *ccbe1* expression was retained in *cloche*^{t22499} mutant embryos (Figure 3D-E) and was therefore non-

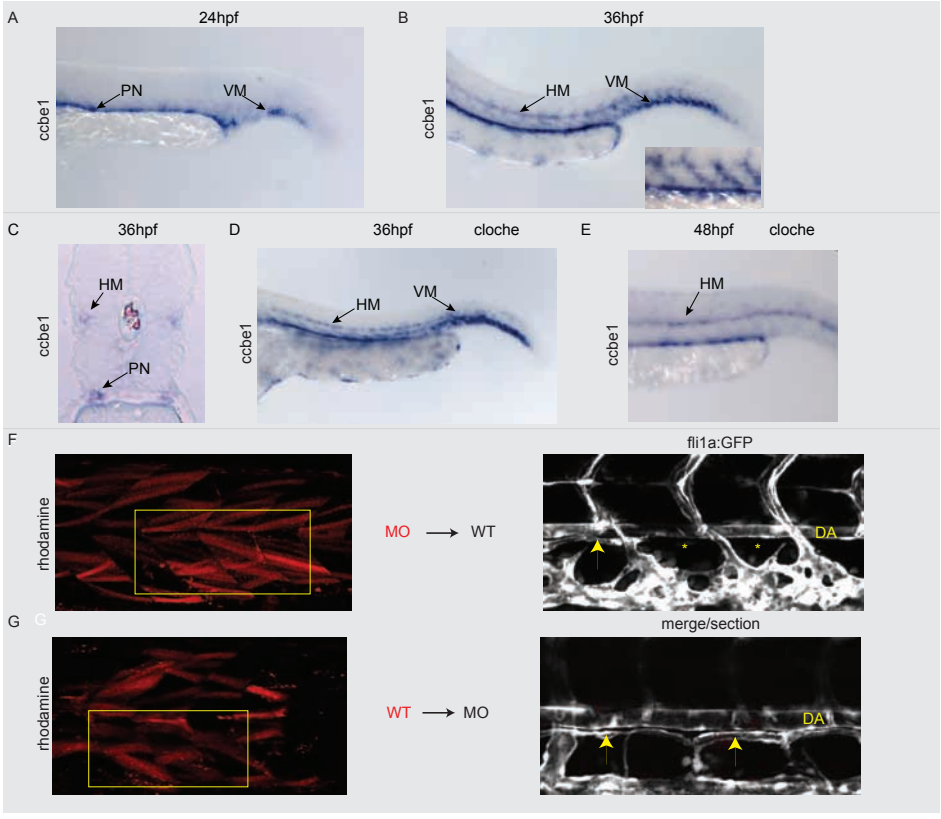

Figure 3: *ccbe1* is expressed within somitic mesoderm and required for non-autonomously for lymphangiogenesis. (A-E) *ccbe1* trunk expression is restricted to ventral mesenchyme (vm) and pronephros (pn) at 24 hpf. At 36 hpf, expression extends along somitic boundaries (inset) and the horizontal myoseptum (hm) (bilateral in section). *ccbe1* trunk expression is restricted to the horizontal myoseptum at 48 hpf when lymphangioblasts populate this region. F. Morpholino injected cells (rhodamine labeled, left) transplanted into wildtype embryos disrupt thoracic duct formation (GFP, right, boxed area) when contributing to somitic mesoderm. In a total of 92 transplants, n=7/7 disrupted thoracic ducts were immediately ventral to labeled muscle cells (5dpf). G. Wildtype donor cells (rhodamine labeled, left) rescued thoracic duct formation by morphant cells (GFP/rhodamine in single confocal section, right, boxed area) when contributing to somatic mesoderm. n=134 embryos transplanted, n=7/7 with lymphatic fragments immediately ventral to grafted muscle (see supplementary Figure 4).

endothelial. Human *CCBE1* is also not expressed in blood or lymphatic endothelial cells (microarray analysis; H. Augustin, personal communication, DKFZ, Heidelberg). Notably, this dynamic non-endothelial expression pattern correlates spatially and temporally with the migration routes of endothelial cells that bud from the PCV, migrate in association with somite boundaries and seed the horizontal myoseptum region from where lymphatic precursors later migrate (Isogai et al., 2003; Yaniv et al., 2006). Using embryonic transplantation assays we found that *ccbe1* function was indeed required in somitic mesoderm for adjacent thoracic duct formation and was sufficient for thoracic duct formation in otherwise *ccbe1*-deficient embryos when restricted to somitic mesoderm Figure 3F,G) Taken together, the domain structure of Ccbe1 (signal peptide, collagen domain, calcium-binding EGF domain), its non-endothelial expression and its somitic mesodermal role indicate that *ccbe1* acts non-autonomously during embryonic lymphangiogenesis.

To directly observe embryonic lymphangiogenesis, we produced a transgenic line by using the promoter of *stabilin1*, a gene expressed in the same manner as *lyve1* (Banerji et al., 1999) in zebrafish (Supplementary Figure 5 online) and a marker of lymphatics in mammals (Prevo et al., 2004; Salmi et al., 2004). Expression of *stabilin1:YFP* was enriched in the PCV and venous-derived endothelial cells, including endothelial cells at the horizontal myoseptum by 48 hpf. At later stages, expression was largely restricted to lymphatic and venous endothelium (Figure 4A). Using live imaging, we observed that *stabilin1:YFP*-positive cells bud directly from the PCV to the horizontal myoseptum from approximately 36 hpf (Supplementary Movie 1 online). Between 48 and 72 hpf, these cells remained in this region before migrating dorsally, to contribute to the DLLV, or ventrally, to contribute to the thoracic duct (Supplementary Movie 2 online). In *ccbe1* morphants, *stabilin1:YFP*-expressing cells failed to bud from the PCV (Figure 4A and Supplementary Movie 3 online).

Vegfc–Vegfr3 signaling is required for the initiation of lymphangioblast budding in mammals (Karkkainen et al., 2004; Makinen et al., 2001). Zebrafish *vegf-c* but not *vegf-d* expression is found immediately dorsal to the PCV before and concurrent with the budding of *stabilin1:YFP*-expressing cells (Supplementary Figure 5)(Covassin et al., 2006). We injected *vegf-c* targeting morpholinos (Kuchler et al., 2006) and found that *vegf-c* was required for budding of *stabilin1:YFP* -positive cells at precisely the stage requiring *ccbe1* (Figure 4A and Supplementary Movie 4 online).

As observed for *stabilin1:YFP*-expressing cells, previous studies have described budding of cells from the PCV to the horizontal myoseptum to form a structure dubbed the parachordal vessel (Isogai et al., 2003) from which lymphatic precursors later derive (Yaniv et al., 2006). We found that the *fli1:GFP*-expressing cells in the parachordal region do not form a vascular tube or support blood flow (Figure 4B). Rather, by taking advantage of the absence of *kdr-like (flk1)*(Bussmann et al., 2008) expression in lymphatic vessels (Supplementary Figure 6 online), we found that the majority of these cells migrate away from the horizontal myoseptum to contribute to the lymphatic vasculature and are present only transiently at the horizontal myoseptum during development (Figure

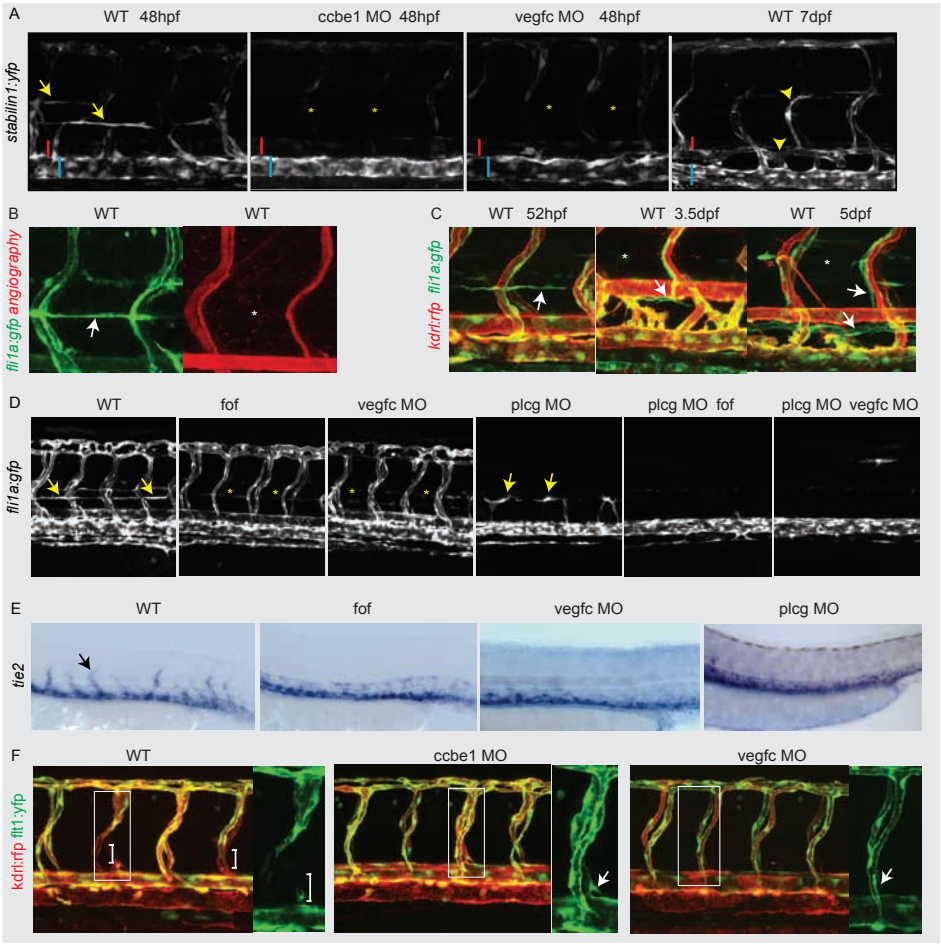

Figure 4: *ccbe1* and *vegf-c* are required for lymphangioblast budding and angiogenic sprouting from venous endothelium. A. *stabilin1:YFP* expression marks the PCV (blue bars, PCV; red bars, DA) and derived lymphangioblasts (arrows indicate bilateral cells) at 48 hpf, and the lymphatic vasculature (arrowheads) and PCV at 7 dpf, with progressively weakening arterial expression. *stabilin1:YFP*-expressing cells fail to bud in *ccbe1* (*n* = 21/22) and *vegf-c* morphants (*n* = 29/31) (48hpf). (B) Endothelial cells in the region of the horizontal myoseptum express *fli1:GFP* (arrow) but do not sustain blood flow (asterisk, angiogram in red) (52 hpf). (C) *fli1:GFP*-positive lymphangioblasts in the region of the horizontal myospetum are migratory and contribute to the lymphatic vasculature as visualized in *kdr-l:RFP, fli1:GFP* double-transgenic animals (arrows indicate lymphangioblasts (52 hpf and 3.5 dpf) and lymphatic vessels (5 dpf), asterisk indicates absence of parachordal endothelial cells) (see also Supplementary Movie 5). (D) Lymphangioblasts (*fli1:GFP*) fail to bud and migrate in *fof* mutants (*n* = 12/12), *vegf-c* morphants (*n* = 16/16), *fof/plcg* double morphants (*n* = 28/28) and *vegf-c/plcg* double morphants (*n* = 74/83) but not in *plcg* single morphants (*n* = 21/21) at 48 hpf. Arrows indicate lymphangioblasts, asterisk indicate their absence. (E) *tie2* is expressed in PCV sprouts in wild-type embryos which are absent in *fof* mutants (*n* = 35/35), *vegf-c* morphants (*n* = 57/64) or *plcg* morphants (*n* = 55/55) at 48 hpf. (F) In wild-type embryos, venous sprouts are *flt1:YFP* negative at 48 hpf in *kdr-l:RFP, flt1:YFP* double transgenic embryos. In *ccbe1* (*n* = 20/20) and *vegf-c* morphants (*n* = 22/25), venous sprouts are absent and in their absence a functional circulation is established due to increased ISV connections with the dorsal aorta. Bracket indicates venous derived sprouts, arrows indicate arterial connections.

2C and Supplementary Movie 5 online). Therefore, these cells do not constitute a blood vessel but rather a population of parachordal lymphangioblasts (PLs).

We next examined *fli1:GFP*-expressing lymphangioblasts in the absence of arteries, a scenario experimentally induced by suppressing *plcg* (*phospholipase C gamma 1*) function (Lawson et al., 2003). In *plcg* morphants, *fli1:GFP*-expressing lymphangioblasts budded from the PCV in the absence of intersegmental vessels (ISVs), but budding was absent in *fof* mutant and *vegf-c* morphant embryos (Figure 4D). *tie2* expression identifies venous derived sprouts in wild-type embryos (48 hpf), which were absent in *fof* mutants and *vegf-c* morphants. However, these sprouts were also absent in *plcg* morphants, indicating that they are a cellular population distinct from lymphangioblasts (Figure 4E). Hence, we developed a double transgenic line, Tg*(flt1:YFP, kdr-l:RFP)*, which unmasks the venous or arterial origins of individual cells of the trunk vasculature. Venous-derived ISV sprouts were absent in *ccbe1* and *vegf-c* morphants (Figure 4F). In the absence of venous-derived ISV sprouts, larvae maintained a functional circulation without blood pooling or hemorrhaging, although we did observe reduced blood flow in some ISVs (data not shown). Hence, both angiogenic sprouting and lymphangiogenic budding from the PCV require *ccbe1* and *vegf-c*, but only angiogenic sprouting requires *plcg*, genetically separating the two processes.

Taken together, these data demonstrate that, during embryogenesis, *ccbe1* and *vegf-c* act at the level of both angiogenic sprouting and lymphangiogenic budding from venous endothelium. *ccbe1* is necessary for lymphangiogenesis, but we find no genetic evidence that it is a component of either the *vegfc–vegfr3* signaling or *sox18–prox1* transcriptional pathways (Supplementary Table 2 online). We suggest that Ccbe1 defines an independent regulator of lymphangioblast budding (and possibly migration), which might work as an extracellular guidance molecule. Alongside *vegf-c* (Karkkainen et al., 2004), *prox1* (Wigle et al., 2002; Wigle and Oliver, 1999) and *sox18* (Francois et al., 2008) deficient mice, the *full of fluid* mutant represents just the fourth vertebrate mutant that leads to the specific loss of the embryonic lymphatic vasculature.

MATERIALS AND METHODS

Zebrafish strains and screen

All zebrafish strains were maintained in the Hubrecht Institute using standard husbandry conditions. Animal experiments were approved by the Animal Experimentation Committee (DEC) of the Royal Netherlands Academy of Arts and Sciences. Published transgenic lines used were Tg(*fli1a:gfp*)^{y1}(Lawson and Weinstein, 2002b). The *cloche*^{t22499} allele has been previously described(Herpers et al., 2008). ENU mutagenesis was performed as previously described for the creation of the Hubrecht Institute target selected mutagenesis library (Wienholds et al., 2002). F1 progeny of mutagenised males were outcrossed to the fli1:GFP strain to create approximately 300

F2 families, which were then incrossed. F3 progeny were screened for the presence of the thoracic duct.

Genetic mapping and genotyping

Bioinformatic construction of the genomic region surrounding the ccbe1 gene was performed using the Ensembl database (http://www.ensembl.org), release 44, April 2007. Meiotic mapping of the full of fluid mutation was performed using standard simple sequence length polymorphisms. The primers used for SSLP and SNP markers depicted in Fig. 1 can be found in Supplementary Table 1. Total RNA (1µg) from wildtype and mutant embryos was reverse transcribed using 12.5µMrandom hexamers, 8mM MgCl2, 1mM each dNTP, 1U/µl RNase inhibitor, and 10U/µl MMuLV reverse transcriptase (Promega). PCR followed by sequencing of wildtype and mutant cDNA was performed with the primer pairs, ccbe1-cDNA f1/r1, *ccbe1*-cDNA f2/r2 and *ccbe1*-cDNA f3/r3 (Supplementary Table 1).

Subsequent genotyping of *ccbe1* mutants was performed on individual embryos by utilizing aninformative *ccbe1* intronic CA repeat marker with the primer pair *ccbe1*-intron CA (Supplementary Table 1).

Morpholino oligos and mRNA synthesis

The *ccbe1* start codon targeting MO (Genetools, LLC) was injected at a concentration of 2.5 or 5ng/embryo. The *ccbe1* splice site targeting MO (Genetools, LLC) was injected at a concentration of 5ng/embryo. The *plcg* MO (Open biosystems) was injected at a concentration of 10ng/embryo. The vegf-c MO (Genetools, LLC) has been previously described (Kuchler et al., 2006; Ober et al., 2004)and was injected at a concentration of 2.5ng/embryo. The *prox1* MO (Genetools, LLC) has been previously described (Kuchler et al. 2006) and was injected at concentrations of 5, 2 and 1ng/embryo. All morpholino oligo sequences are given in Supplementary Table 1. The full length ccbe1 coding sequence was PCR amplified from the template EST clone EE696184 using the primer pair *ccbe1*-CDS f/r (Supplementary Table 1) and cloned into pCS2+ using the EcoRI and XhoI restriction sites. Mutagenesis of the ccbe1 pCS2+ clone was performed using QuikChange site-directed mutagenesis (Stratagene) with the amplification primer pair ccbe1-D162E f/r (Supplementary Table 1). *Vegf-c* and *vegf-d* mRNA was made by first cloning the respective coding sequences into the pCS2+ vector using PCR products from a cDNA template clone (vegf-c) (Open Biosystems) or cDNA (vegfd) with the primer pairs *vegfc*-CDS f/r and *vegfd*-CDS f/r (Supplementary Table 1), followed by cloning into EcoRI and XhoI restriction sites. In vitro transcription was performed from NotI digested template using the SP6 mMessage mMachine kit for all injected mRNA (Ambion). *ccbe1* mRNA was injected at 400pg/embryo with lower doses failing to achieve rescue.

Generation of transgenic lines

The Tg(kdr-l:ras-cherry)^{s916} line was generated by using a construct containing a 6.8kb fragment of the kdr-like (Bussmann et al., 2008)promoter (Beis et al., 2005) upstream of a ras-cherry fusion element. We injected 200 pg of linearized DNA into one cell-stage embryos and selected individual transgenic carrier adults by screening for fluorescent progeny.

For *stabilin1:YFP* and *flt1:YFP* transgenic lines, a citrine-neomycin cassette was recombined using Red/ET Recombination Technology (Gene Bridges) into the BAC (bacterial artificial chromosome) clones DKEY-182E1 (stabilin1) or DKEY-256I8 (flt1) using the homology arm tagged PCR primers, stabilin1 f/r and flt1 f/r (Supplementary Table 1). In addition, an ISceI meganuclease site was integrated into the clone backbone for ease of transgenesis aspreviously described(Kimura et al., 2006). DNA was injected into one cell-stage embryos at a concentration of 500pg/embryo and selected Tg(*flt1:YFP*)^{hu4624} and Tg(*stabilin1:YFP*)^{hu4453} transgenic carrier adults were derived by screening for fluorescent progeny.

In situ hybridization

In situ hybridization was performed as previously described(Bussmann et al., 2007). The *tie2, vegfr3, sox18* and *prox1* probes have been previously described (Herpers et al., 2008; Lyons et al., 1998; Thompson et al., 1998). *ccbe1* probe was synthesized by in vitro transcription from the EcoRI-digested full length cDNA in pCS2+ using T7 RNA polymerase (Promega). *Vegf-c* and v*egf-d* riboprobes were made from EcoRI-digested template (details above) using the T7 RNA polymerase (Promega). lyve-1 probe clone (corresponding to ENSDARG00000062483) was a gift from Exelixis Inc. RNA was synthesized by digestion with EcoRV and transcription with SP6 polymerase. *stabilin-1* probe was synthesized by first PCR-amplifying an exonic fragment with the PCR primer pair stabilin1-probe f/r (Supplementary Table 1) and then synthesizing RNA using T3 RNA polymerase (Promega).

Genetic interaction experiments

To test if ccbe1 heterozygosity sensitized embryos to low dose injections of a soluble dominant negative form of vegfr35, embryos derived from a heterozygous outcross (fof +/- x fof +/+) were injected with a sub-critical dose of sVegfr3 mRNA. Embryos that displayed a severe reduction or loss of thoracic duct formation were then genotyped for the fof mutation (expected ratio of fof +/-: fof +/+.genotypes would be 1:1 (50% heterozygous) if no interaction were observed). The identical experimental procedure was used to examine the potential for *prox1* to genetically interact with ccbe1, however, prox1 MO injection led to cardiac edema phenotypes (as previously described4) which precluded an analysis of thoracic duct formation. The ability of *vegf-c* or v*egf-d* mRNA to rescue fof was tested by injecting mRNA into a *fof* heterozygote incross and subsequently genotyping embryos that were positive for thoracic duct development to determine if mutant rescue was achieved. The ability of ccbe1 mRNA to rescue the

vegf-c MO induced loss of the thoracic duct was tested by injecting ccbe1 mRNA into vegf-c morphants.

Imaging

Embryos were mounted in 0.5-1% low melting point agarose in a culture dish with a cover slip replacing the bottom. Imaging was performed with a Leica SP2 or SP5 confocal microscope (Leica Microsystems, http://www.leica-microsystems.com/) using a 10x, 20x or 40x objective with digital zoom. Timelapse analysis was compiled using ImageJ software (http://rsb.info.nih.gov/ij/). Time points were recorded every 20 minutes for the stated time period. A heated stage maintained the embryos at approximately 28.5 °C.

Transplantation

Transplantation was performed essentially as previously described (Ho and Kane, 1990; Hogan et al., 2008). Briefly, wildtype donor embryos of the genotype Tg(fli1:GFP) were injected with 70KDa Tetramethyl Rhodamine (TAMRA) (Molecular Probes) with or without ccbe1 ATG MO (5ng/embryo) at the one cell-stage and utilized as donors at pre-dome stages. More than 10 cells were transferred from donor to recipient embryos between sphere and 30% epiboly stages. Embryos were first scored for the presence or absence of lymphatic fragments in the thoracic duct region and then analysed for relative grafted cell position. Only morphologically normal embryos were examined.

Accession numbers

Accession numbers for clones used in this study are as follows: *ccbe1* clone, EE696184; *vegf-c* clone, BC114253.

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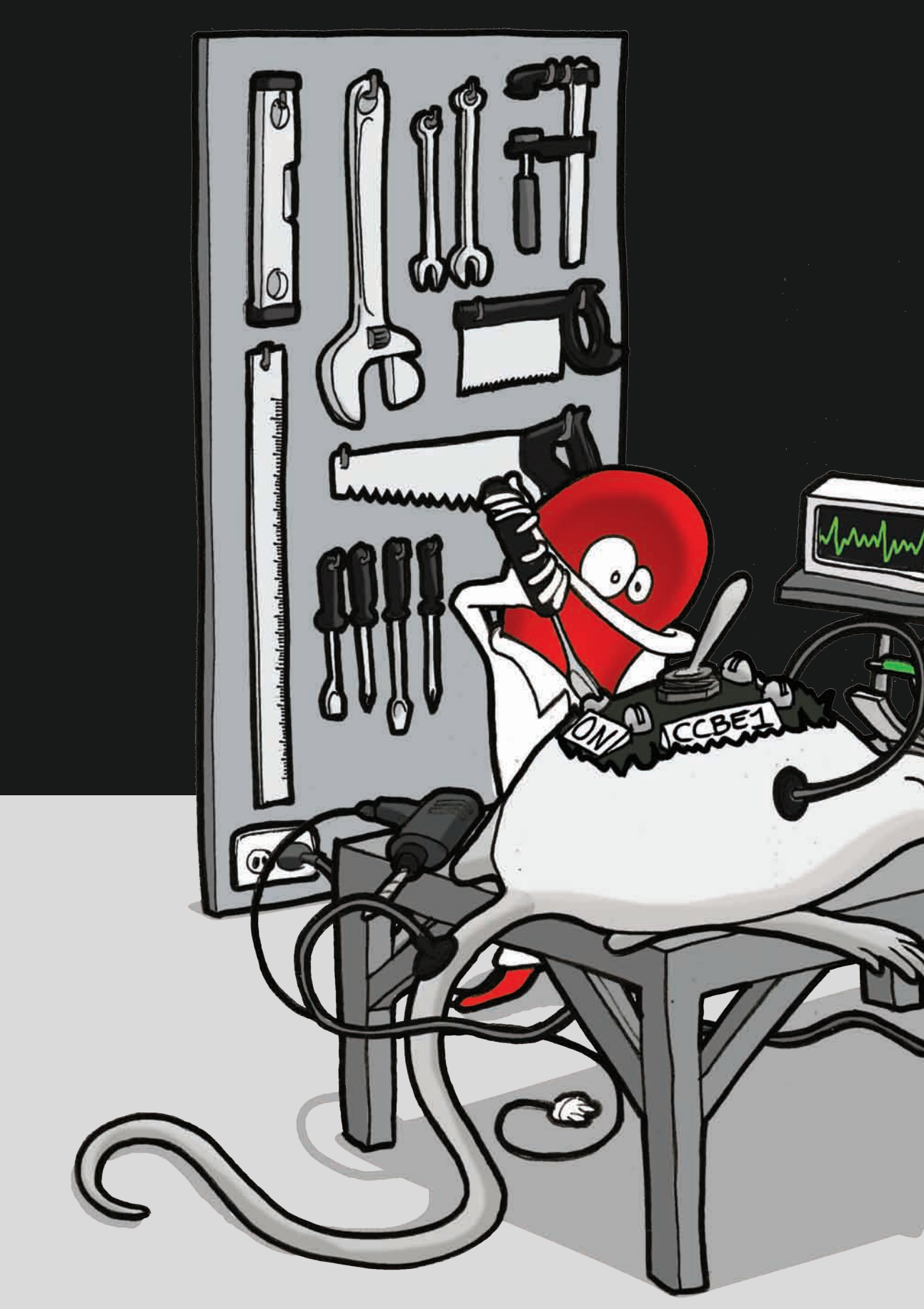
AUTHOR CONTRIBUTIONS

B.M.H. conceived, carried out experiments and co-wrote manuscript. F.L.B., J.B., N.C.C. and M.W. conceived and carried out experiments. H.J.D. conceived experiments. S.S.M. conceived experiments and co-wrote the manuscript

SUPPLEMENTARY INFORMATION

Available on the Nature Genetics website. http://www.nature.com/ng/journal/v41/n4/suppinfo/ng.321_S1.html


ON
CCBE1

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CCBE1 IS ESSENTIAL FOR MAMMALIAN LYMPHANGIOGENESIS AND ENHANCES THE EFFECT OF VEGF-C IN VIVO

Frank L. Bos[1,2], Maresa Caunt[3], Josi Peterson-Maduro[1], Lara Planas-Paz[4], Joe Kowalski[3], Terhi Karpanen[1], Andreas van Impel[1], Raymond Tong[5], James A. Ernst[5], Jeroen Korving[1], Johan H. van Es[1], Eckhard Lammert[4], Henricus J. Duckers[2] & Stefan Schulte-Merker[1,6]

1. Hubrecht Institute – Koninklijke Nederlandse Akademie van Wetenschappen and University Medical Centre Utrecht, 3584 CT Utrecht, The Netherlands.
2. Erasmus Medical Centre Rotterdam, 3015 GE Rotterdam, The Netherlands.
3. Molecular Biology Department, Genentech Inc, CA 94080-94990, South San Francisco, USA.
4. Institute of Metabolic Physiology, Heinrich Heine Universität Düsseldorf, 40225 Düsseldorf, Germany.
5. Protein Chemistry Department, Genentech Inc, CA 94080-94990, South San Francisco, USA.
6. Experimental Zoology Department, University of Wageningen, 6709 PG Wageningen, The Netherlands

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SUMMARY

Lymphatic vessels are crucial for tissue fluid homeostasis, immune cell trafficking and absorption of dietary lipids. Here we show the secreted factor collagen and calcium-binding EGF domains 1 (Ccbe1) to be required for the primary sprouting of lymphatic endothelial cells (LECs) from murine embryonic veins. We have generated *Ccbe1*$^{-/-}$ mice that exhibit a lack all lymphatic vessels and that die prematurely with severe edema. However, initial LEC differentiation in venous structures of these mice does occur, and vascular endothelial growth factor receptor 3 (VEGFR-3) activation appears unaltered. Moreover, we demonstrate that human CCBE1 protein binds to components of the extracellular matrix, and that exogenous CCBE1 protein strongly enhances VEGF-C mediated lymphangiogenesis in a cornea micro-pocket assay in vivo. Our data identify Ccbe1 as a secreted factor critically required for mammalian lymphangiogenesis, an essential enhancer of VEGF-C activity, and a potential therapeutic target for modulating lymphatic vascular-associated disorders.

INTRODUCTION

The human vascular system consists of blood and lymphatic vessels, both of which are lined by endothelial cells (ECs)(Oliver and Srinivasan, 2010). Lymphatic vessels play a key role in fluid homeostasis and are central to transport of macromolecules, immune system function, and intestinal lipid absorption (Cueni and Detmar, 2008). The importance of lymphatic vessels is underscored by the fact that mice lacking lymphatic vessels due to loss-of-function mutations in the VEGF-C gene die *in utero* displaying severe edema (Karkkainen et al., 2004). Furthermore, functional defects of lymphatic vessels result in a number of clinical conditions, such as lymphedema (Witte et al., 2001). Lymphatic vessels are also implicated in the metastatic dissemination of tumor cells, and therefore play an important role in tumor progression (Achen et al., 2005). Thus, identifying the molecular mediators of lymphatic vascular development and mechanisms by which they modulate lymphangiogenesis could be of significant clinical importance.

During vertebrate embryogenesis, lymphatic endothelial cells (LECs) originate from veins (Hogan et al., 2009; Ny et al., 2005; Srinivasan et al., 2007). In mice, polar expression of lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) (Jackson et al., 2001) and Prospero homeobox 1 (Prox-1) (Oliver and Harvey, 2002) in a subset of anterior cardinal vein cells indicates LEC fate specification. Prox1-positive cells then migrate away from veins to form lymph sacs, structures that support subsequent lymphatic vessel growth in a process requiring both the chemo-attractant VEGF-C and signaling through the VEGFR3 pathway (Karkkainen et al., 2004; Kukk et al., 1996). To date, genetic studies have been limited to the identification of *Prox1* (Wigle and Oliver, 1999), *Vegfc* (Karkkainen et al., 2004) and *Vegfr3* (Brice et al., 2005; Karkkainen et al., 2000), as the only known essential factors for murine lymphangiogenesis to occur.

Recently, we identified the collagen and calcium binding EGF domains 1 (*ccbe1*) gene to be required for embryonic lymphangiogenesis in zebrafish (Hogan et al., 2009). Furthermore, CCBE1 was found to be mutated in a cohort of patients with Hennekam Syndrome (HS) (Alders et al., 2009), a rare disease that presents with lymphedema, lymphangiectasias, varying degrees of mental retardation, and other pathological features (Connell et al., 2010; Van Balkom et al., 2002). However, since in zebrafish venous sprouting and the generation of lymphatic precursor cells occurs simultaneously (Bussmann et al., 2010), and since early markers distinguishing venous ECs from LECs are lacking, it has remained unclear in which stage of lymphatic development *ccbe1* is required. Furthermore, whether CCBE1 is an instructive or permissive factor has not been resolved.

RESULTS AND DISCUSSION

Generation and characterization of Ccbe1$^{-/-}$ mice

To determine the stage during lymphangiogenesis that requires Ccbe1 and to more precisely define its function during mammalian vascular development, we targeted the murine *Ccbe1* locus by generating both a floxed allele of *Ccbe1* (*Ccbe1loxP)* and a *lacZ* allele (*Ccbe1lacZ)* (Figure 1A, B; also see Methods). We henceforth present data from the *Ccbe1lacZ* mice unless otherwise noted. During embryonic development, cells expressing *Ccbe1* were found in multiple locations such as the brain, the mesothelium of the heart, and in close proximity to nascent blood and lymphatic vascular structures (although *lacZ* expression was weak in some of these regions) (Figure 1C, D). *Ccbe1*$^{-/-}$ embryos were indistinguishable from their wildtype and heterozygous littermates when examined for gross morphology up to E13.5. Based on examination of blood flow and platelet endothelial cell adhesion molecule-1 (PECAM-1) staining at E11.5 and E14.5, patterning and density of blood vessels exhibited no gross abnormalities (Figure 1E and Figure S1 A, B). From E13.5 on, however, *Ccbe1*$^{-/-}$ mice accumulated fluid and developed edema (Figure 1F), suggesting a failure in the lymphatic vascular system. Further analysis revealed that in both strains (*lacZ* and *loxP*), *Ccbe1*$^{-/-}$ embryos lack all lymphatic vessels such as those found in skin, mesenteries (Figure 1 G-I) and diaphragm (not shown). Thus, in mice Ccbe1 appears to be critically important for lymphangiogenesis, but not for angiogenesis. While this is in contrast to zebrafish, where venous sprouting from the cardinal vein is heavily affected, we believe that this apparent difference is due to timing: in zebrafish, sprouts from the posterior cardinal vein give rise to both definitive veins and lymphatic precursor cells (Bussmann et al., 2010) while in mice lymphatic sprouting is a process distinct from vein formation. That notwithstanding, we here demonstrate that the function of ccbe1/Ccbe1 is evolutionary conserved and required for lymphangiogenesis.

Migration of lymphatic endothelial cells from the cardinal vein is impaired in *Ccbe1*$^{-/-}$ mice

The dramatic loss of lymphatic structures raised the possibility of abnormal primary specification of LECs within venous structures. We used LYVE-1 (Jackson, 2003; Oliver, 2004) and Prox-1 (Wigle and Oliver, 1999), two early markers for lymphatic cell fate specification, to address this question. At E10.5, when initial LEC specification occurs, LYVE-1^{+} and Prox-1^{+} LECs were detected in *Ccbe1*$^{-/-}$ embryos, (Figure 2A) suggesting that LEC differentiation is unaffected in the absence of Ccbe1. In normal mice, differentiated LECs bud and migrate away from the anterior cardinal veins between E10.5 and E12.0 (Oliver and Harvey, 2002). To address whether LEC budding and migration were normal in *Ccbe1*$^{-/-}$ embryos; we examined the migratory behavior of LECs from the jugular and cardinal veins. At E11.5, we observed fewer LECs outside the cardinal vein in *Ccbe1*$^{-/-}$ embryos as compared to heterozygous siblings (Figure 2B). Additionally, LECs were not observed in ectopic locations, suggesting that abnormal LEC guidance was unlikely to cause the LEC reduction outside the vein.

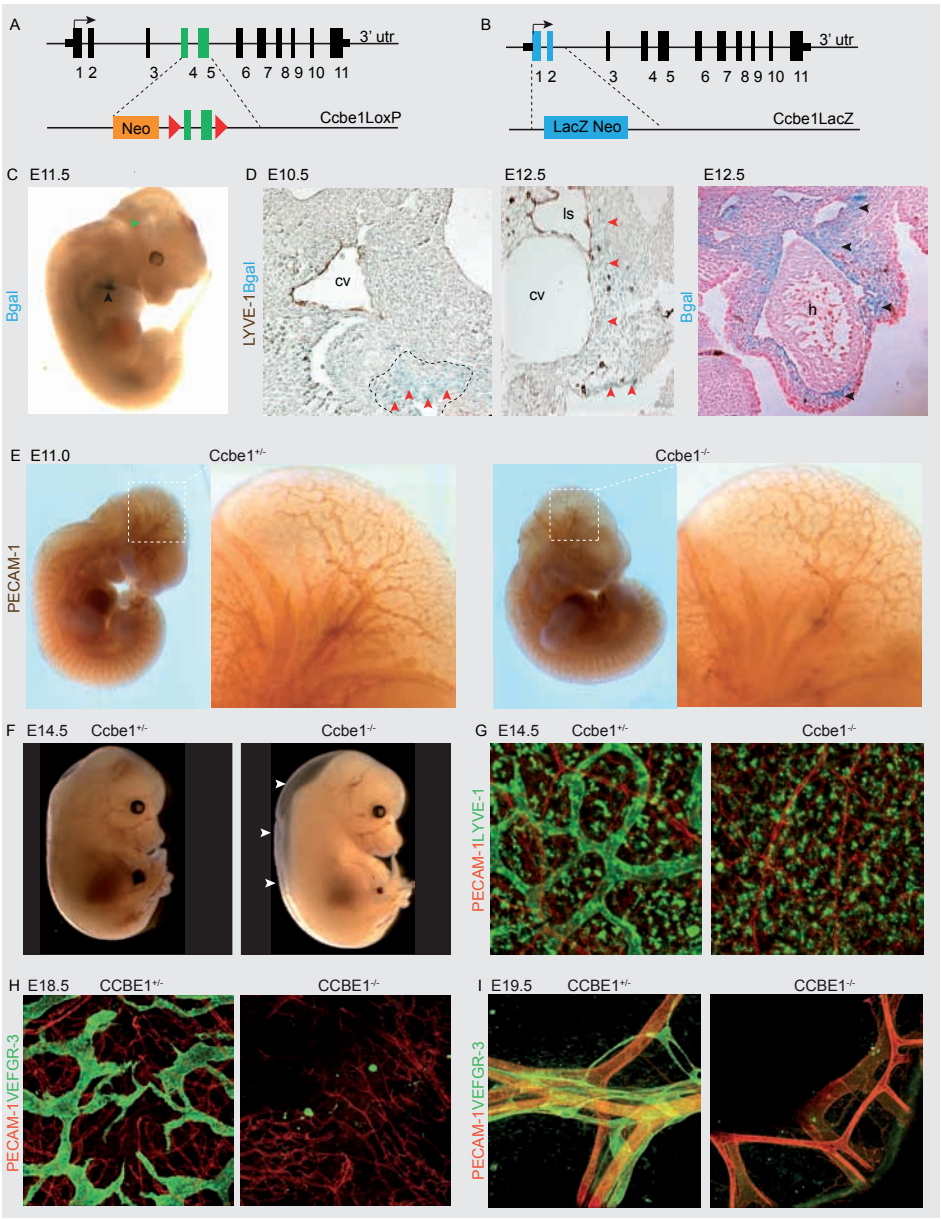

Figure 1: *Ccbe1* mutant mice develop edema and lack lymphatic vessels. A Coding exons 1 and 2 of the *Ccbe1* gene were replaced by *lacZ* cassette (*Ccbe1*^{lacZ}). B Coding exons 4 and 5 of Ccbe1 were targeted by flanking *loxP* sites (*Ccbe1*^{loxP}). Deletion was accomplished by crossing to a generic *PGK:Cre* delete. C. β-galactosidase staining (blue) of an E11.0 *Ccbe1*$^{+/-}$ *lacZ* embryo showing expression surrounding the heart (black arrows) and in the brain (green arrow). D Transverse sections of *Ccbe1*$^{+/-}$ *lacZ* embryo at E10.5 and E12.5; β-galactosidase expression can be detected near vascular structures (red arrows) and mesothelium of the heart (black arrows). E. Whole mount PECAM-1 staining of a *Ccbe1*$^{-/-}$ *lacZ* embryo revealing no abnormalities in blood vascular patterning. F. Edema formation in an E14.5 *Ccbe1*$^{-/-}$ *lacZ* embryo. G. LYVE-1 (green) /PECAM-1 (red) staining of E14.5 *Ccbe1*$^{-/-}$ skin which lack lymphatic vessels but retain LYVE-1^{+} macrophages. H. VEGFR-3 (green) / PECAM-1 (red) staining of E18.5 *Ccbe1*$^{-/-}$ *loxP* skin at midline region which lack lymphatics. I. VEGFR-3 (green) /PECAM-1 (red) staining of E19.5 *Ccbe1*$^{-/-}$ *loxP* mesenteries which lack all lymphatics. CV: cardinal vein, a: aorta, ls: lymph sac.

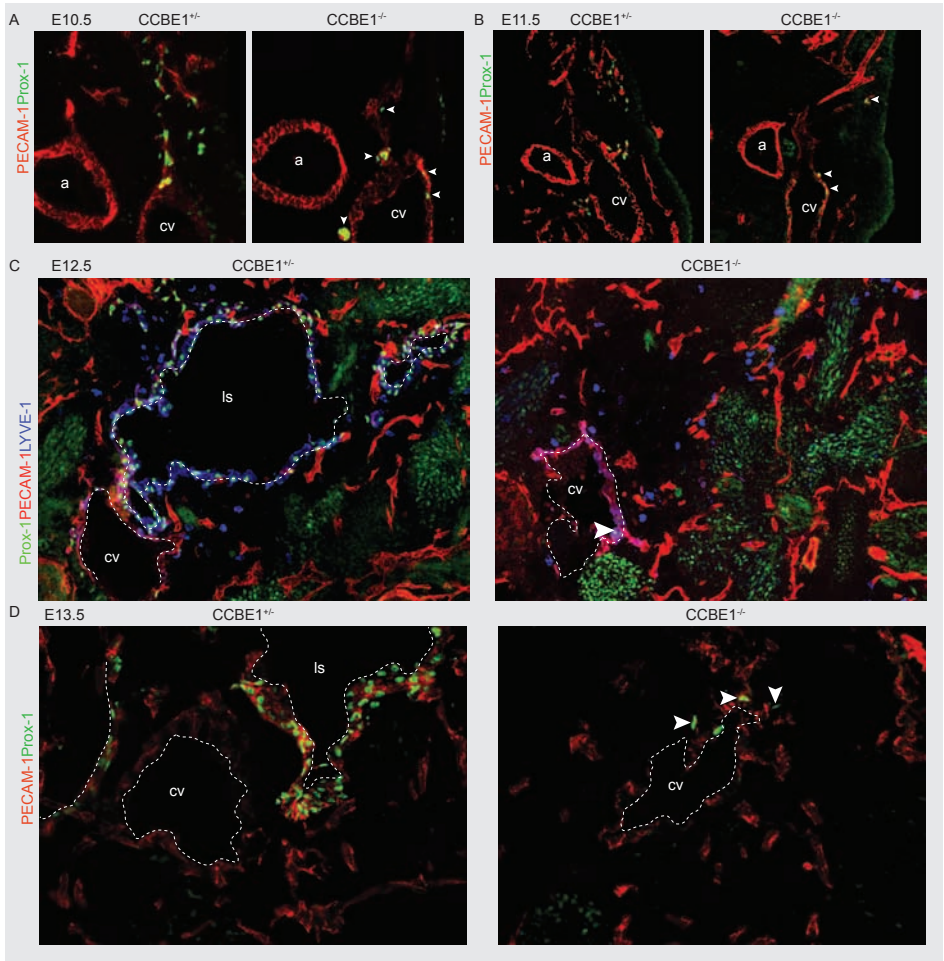

Figure 2*: Ccbe1* deficiency results in failure of lymphatic vascular development. A. Prox-1/PECAM-1 staining at E10.5. Prox1 expressing LECs are present and specified in the cardinal vein of both siblings and mutants, but LEC number are reduced in mutants. B. Prox-1/PECAM-1 staining at E11.5. In *Ccbe1*$^{+/-}$ siblings very few cells are still retained in the cardinal vein, the majority of cells have migrated out. In *Ccbe1* mutants, fewer Prox1^{+} cells are detectable overall, and the vast majority of these cells are retained at the level of the cardinal vein (arrowheads). C. Prox-1/PECAM-1/LYVE-1 staining of E12.5 embryos. Residual LYVE-1 (pink) and Prox-1 (arrowhead) staining can be detected in the cardinal vein, whereas the lymph sacs are completely absent in *Ccbe1*$^{-/-}$ embryos. D. Prox-1/PECAM-1 double staining of the cardinal vein region at E13.5. In *Ccbe1*$^{+/-}$ siblings all Prox-1^{+} cells have migrated away (arrowheads) from the cardinal vein and form lymph sacs, while *Ccbe1*$^{-/-}$ mutants lack Prox1^{+} lymph sacs. Note the small number of PROX1^{+} cells within the cardinal vein. cv: cardinal vein, a: aorta, ls: lymph sac.

Normally, lymph sacs are formed between E11.5 and E13.5, and these subsequently give rise to most of the lymphatic vasculature. We observed no lymph sacs, and only a few Prox-1^{+} and LYVE-1^{+} LECs were present in the wall of the cardinal vein in the *Ccbe1*$^{-/-}$ embryos, indicating an overall reduction in LECs at these stages and a primary

defect prior to the formation of lymph sacs (Figure 2C, D). These findings indicate that Ccbe1 is essential for budding and/or migration of LECs from veins. Importantly, since early markers for lymphatic fate are missing in zebrafish, it has remained unclear when lymphatic fates are established in the absence of *Ccbe1*. Here we show that Prox1+ cells do indeed get specified in *Ccbe1*$^{-/-}$ mutants, and that the phenotype is very similar to what has been reported for *Vegfc*$^{-/-}$ mice (Karkkainen et al., 2004).

VEGFR3 phosphorylation is unaltered in Ccbe1-/- LECs

Migration of LECs has previously been shown to be dependent on Vegfc/Vegfr3 signaling (Veikkola et al., 2001). To evaluate whether Ccbe1 is an activator of this pathway, we analyzed LECs for VEGFR-3 tyrosine phosphorylation (pTYR/VEGFR-3) in situ, using a proximity ligation assay (Jarvius et al., 2007; Soderberg et al., 2006) (Figure 3A, B). We quantified both the number of LYVE-1$^+$ cells and the number of pTYR/VEGFR-3 sites per LYVE-1$^+$ cell at E10.5 and E12.5 and confirmed a reduction in number of LYVE-1$^+$ cells in *Ccbe1*$^{-/-}$ embryos (Figure 3C). However, at critical stages of LEC migration, levels of phosphorylated VEGFR-3 per LYVE-1$^+$ cell were unaltered, indicating that VEGFR-3 activation is not affected by the absence of Ccbe1 (Figure 3D) and suggesting that Ccbe1 may function independently of VEGFR-3 phosphorylation.

CCBE1 protein binds components of the extracellular matrix

Since VEGFR3 phosphorylation appeared normal in *Ccbe1*$^{-/-}$ LECs, and since the two domains in the CCBE1 protein (Ca^{2+} binding EGF, Collagen repeat) are found in many extracellular matrix (ECM) proteins, we considered the possibility that CCBE1 might be part of the ECM, rather than acting as a freely diffusible factor that binds to a cellular receptor directly and exerts instructive function. To test this, we set out to generate purified CCBE1 protein. We were unable to produce full length CCBE1 due to formation of insoluble aggregates, as commonly observed in proteins containing collagen-repeat domains. However, since previous (Pantenburg et al., 2010) and new genetic evidence (Figure S2A, B, C) suggests that the majority of inactivating mutations were present in the predicted EGF domain, we generated a truncated protein (CCBE1$^{\Delta collagen}$-Fc) consisting of the calcium-binding EGF domain fused to an Fc domain, but lacking the collagen repeat domain (Figure 4A). This purified protein was incubated with plated HUVECs to determine binding localizations. CCBE1$^{\Delta collagen}$-Fc appeared to bind to collagen IV deposited by HUVEC based on the immunohistochemical staining, (Figure 4B top panels, arrows), and binding could also occur in cell-free areas (Figure 4B bottom panels). To test this notion, we used the same assay but cells were removed prior to incubating the remaining ECM with either recombinant CCBE1$^{\Delta collagen}$-Fc, and either human Fc-containing proteins Alk1-Fc (Niessen et al., 2010) or anti-Nrp2b antibody (Xu et al., 2010) as negative controls. Detection with the respective antibodies revealed that collagen IV (Figure 4C, top panels, arrows), but not laminin (data not shown), co-localized with CCBE1$^{\Delta collagen}$-Fc. Control proteins anti-Nrp2 and Alk1-Fc (data not shown) did not show binding to ECM components (Figure 4C, bottom panels)

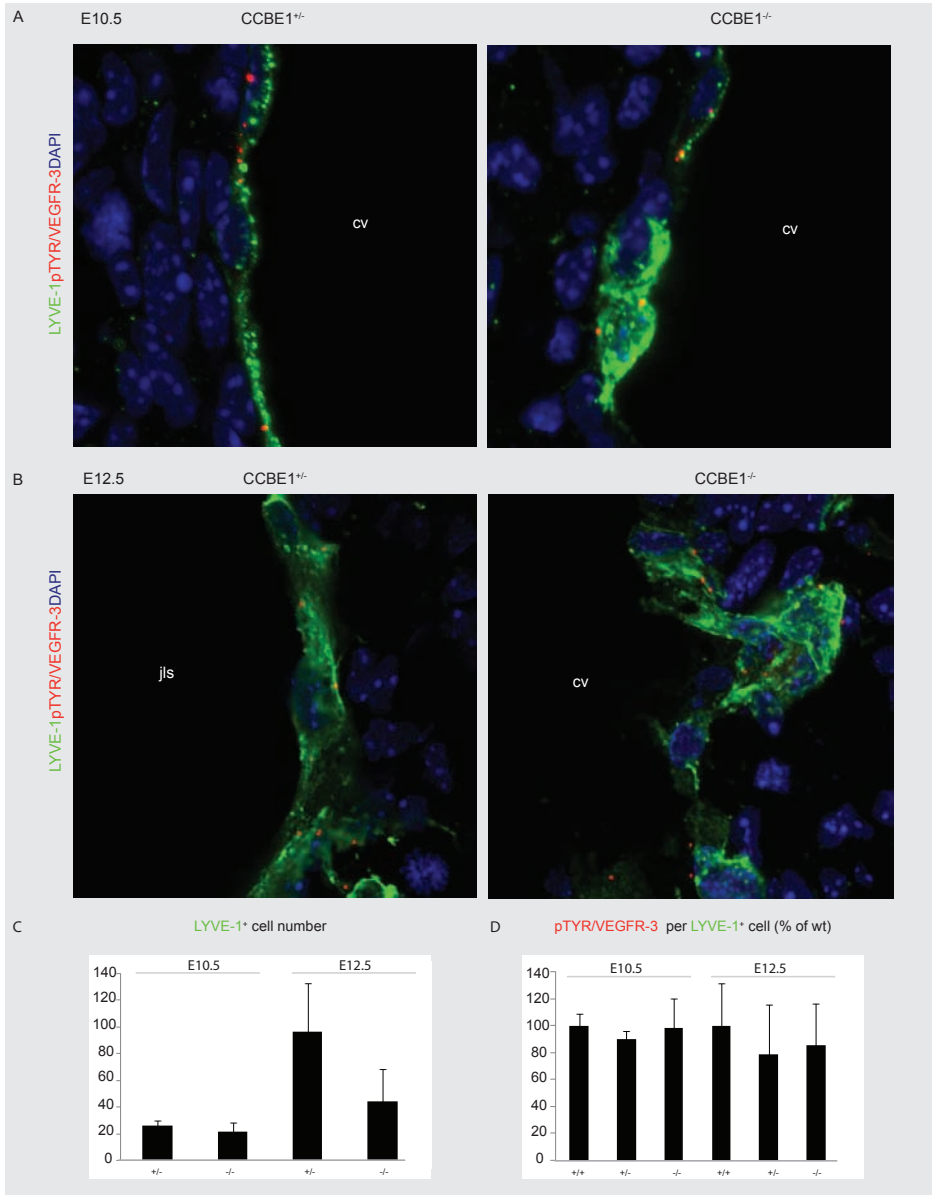

Figure 3: VEGFR-3 tyrosine phosphorylation is unaltered in *Ccbe1* deficient embryos. VEGFR-3 and phosphorylation of Tyrosine (pTYR, red) were detected by in situ proximity ligation assays at E10.5 (A) and E12.5 (B) in LYVE-1$^+$ cells (green). At E10.5 LYVE-1+ (green) cells in both sibling and mutant are still located within the cardinal vein, while at E12.5 LYVE-1+ (green) cells are found in the lymph sacs of sibling embryos. (C). While fewer LYVE-1$^+$ cells were present in *Ccbe1*$^{-/-}$ embryos at E12.5 compared to E10.5. Normalizing per LYVE-1+ cell revealed that the total amount of phosphorylated pTYR/VEGFR-3 per LYVE-1$^+$ cell is unaltered in *Ccbe1*$^{-/-}$ embryos at E10.5 and E12.5 in comparison to sibling embryos (D). cv: cardinal vein, jls: jugular lymph sac.

Since it is possible that the co-localization of CCBE1$^{\Delta collagen}$-Fc with ECM components is indirect (for example because collagen IV and CCBE1 bind a third, independent component of the ECM), we turned to a binary system where plates coated with a single ECM protein were incubated with CCBE1$^{\Delta collagen}$-Fc protein. Here, any binding would suggest the interaction to be direct. We found reproducible but moderate interaction of CCBE1$^{\Delta collagen}$-Fc with collagen I, collagen IV and collagen V (Figure 4D), but not with fibronectin and laminin. Strongest binding, however, was reproducibly observed with vitronectin in a dose-dependent manner (Figure 4D). We conclude that the Ca^{2+}-binding EGF domain of CCBE1 can bind vitronectin and other components of the ECM, in particular certain types of collagen. Since we use a truncated form of CCBE1, one might speculate on the function of full-length CCBE1 protein. It is possible that full length CCBE1 protein strengthens the ECM interaction (with the collagen domains), thus fulfilling its function as a mediator of lymphangiogenesis. Strikingly, the truncated protein (without collagen domains) binds to the ECM and it is therefore likely that the Ca^{2+}-binding-EGF domain is also required for the ECM interaction.

CCBE1 strongly enhances the activity of VEGF-C in the corneal micropocket assay

Our analysis of *Ccbe1* $^{-/-}$ mice demonstrates that Ccbe1 is important for LEC budding, migration and sprouting, and our *in vitro* data (Figure 4) suggests that CCBE1 protein presented from the ECM may therefore mediate or permit the chemoattractive effect of VEGF-C. We hence tested whether exogenous CCBE1 may modify the lymphangiogenesis activity of VEGF-C *in vivo*. To this end, the CCBE1$^{\Delta collagen}$-Fc protein was employed in a corneal micro-pocket assay (Caunt et al., 2008; Kubo et al., 2002) and its effect compared to the effects of VEGF-C, a known inducer of lymphangiogenesis in the avascular cornea of adult mice. When examined after 10 days, a single administration of CCBE1$^{\Delta collagen}$-Fc weakly but significantly induced growth of lymphatic vessels from the limbus region underneath the implanted pellets, whereas VEGF-C elicited a strong response (Figure 5A, C). Strikingly, the lymphangiogenic response was strongly increased when CCBE1$^{\Delta collagen}$-Fc was administered together with VEGF-C, yielding a greater effect than previously observed with any other protein (Figure 5A, C). These findings suggest that CCBE1 is a strong positive modulator of lymphangiogenesis and that the Ca^{2+}-binding EGF domain is sufficient to elicit this response. Of note, lymphatic vessels induced by the combined activity of CCBE1 and VEGF-C show a dramatically increased number of filopodial extensions in comparison to vessels induced by VEGF-C alone (Figure 5B, D). This finding is consistent with our results that CCBE1 plays a role in LEC sprouting and budding.

Since the application of CCBE1 alone causes a weak response of lymphatic vessel outgrowth, this might be taken as an argument that CCBE1 is a molecule facilitating LEC chemotaxis. It has been shown that the infliction of a wound in the cornea is sufficient to recruit macrophages to the site of injury, which are known to produce high levels of VEGFC (Schoppmann et al., 2002). Hence, it is conceivable that applying

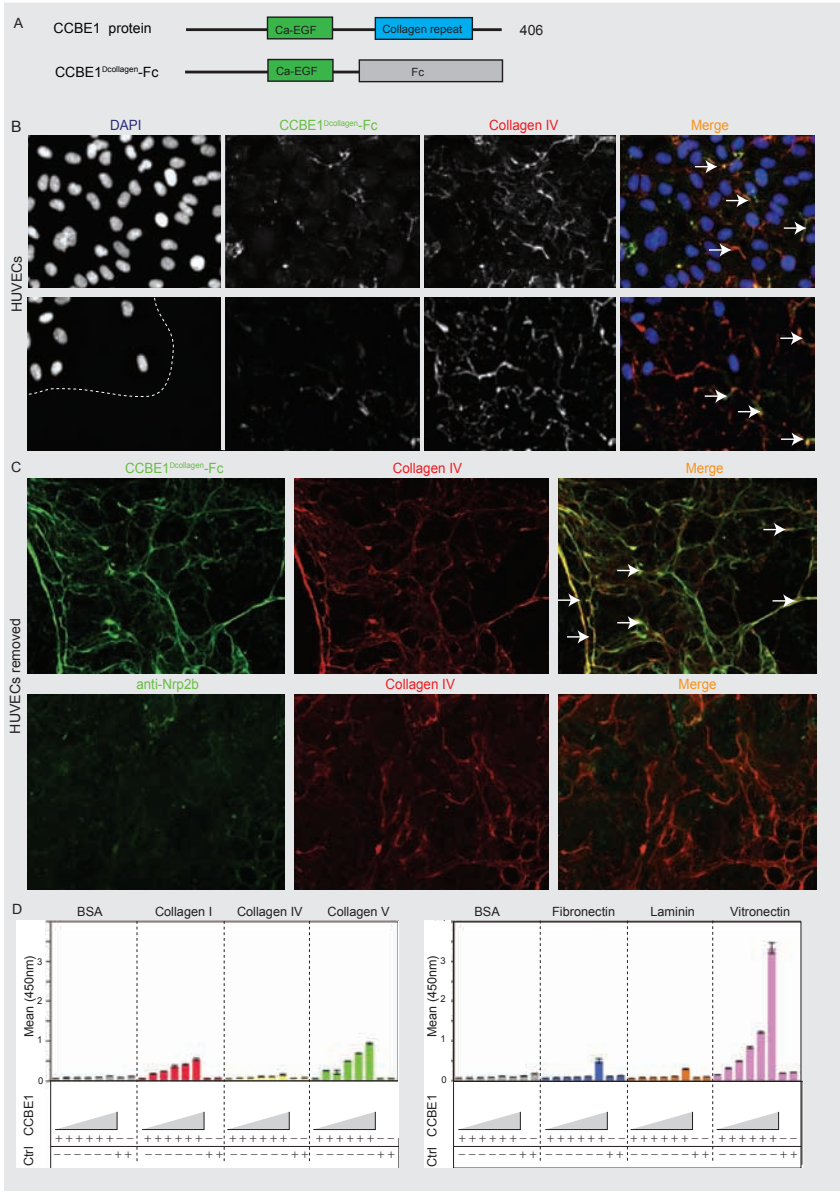

Figure 4: Ccbe1 binds to components of the extracellular matrix. A. Schematic representation of wildtype and recombinant CCBE1$^{\Delta collagen}$-Fc protein. B. CCBE1$^{\Delta collagen}$-Fc (green) binds to basal side of HUVECs (dapi, bleu) and ECM component Collagen IV (red). Co-localization shown in orange (arrowheads). C. After removal of cells, CCBE1$^{\Delta collagen}$-Fc binds to ECM which has been deposited by HUVECs, showing specific co-localization (orange) with Collagen IV (red). Anti-Nrp2 (green) was used as a control and does not bind to Collagen IV (red). D. ELISA of CCBE1$^{\Delta collagen}$-Fc (+) binding to different ECM components: Collagen I, Collagen IV, Collagen V, laminin, fibronectin and vitronectin. CCBE1$^{\Delta collagen}$-Fc binds strongly to vitronectin in a dose-respondent manner. CCBE1$^{\Delta collagen}$-Fc is able to bind to all types of Collagen, but does not bind to laminin and fibronectin. Anti-Nrp2 (-) was used as a control and does not bind to ECM components.

CCBE1 in this context is sufficient to elicit a lymphangiogenic response, during which low levels of macrophage-produced VEGF-C are sufficient to stimulate LECs in a CCBE1-rich environment. Further stimulation with exogenous VEGF-C (Figure 5A, C) will then cause an even stronger response.

We have shown that *Ccbe1* is required for mammalian embryonic lymphangiogenesis, independent of VEGFR-3 phosphorylation. Given the lethal murine *Ccbe1*$^{-/-}$ phenotype, mutations in *CCBE1* in human HS patients are likely to represent severe hypomorphic, but not complete loss-of-function situations. Recently, stillborn HS cases were reported (Connell et al., 2010). Many of the human mutations and all three available zebrafish alleles harbor mutations in, or close to, the calcium-binding EGF domain, identifying this domain as a key functional component of the protein (Figure S2). Supporting the functional importance of the Ca^{2+}-binding EGF domain, a truncated form of CCBE1 containing this domain is sufficient to induce a strong lymphangiogenic response in conjunction with VEGF-C in an *in vivo* assay.

Given the essential role of CCBE1 in lymphatic development one might speculate on its mechanism of action and therapeutic potential. Since Ccbe1 is not required to alter the VEGFR3 activation status, and since the effects of CCBE1 are less dramatic in the cornea pocket assay than the effects of VEGF-C, we favor a model where CCBE1 is not an instructive factor but rather acts as an essentially required permissive factor. As such, CCBE1 is part of, or binds to, the ECM and modulates the effect of VEGF-C on lymphangiogenesis, possibly through vitronectin which is known to affect cell sprouting and migration (Smith and Marshall, 2010).

It has been shown that modulating lymphangiogenesis by growth factor treatment (VEGF-C/VEGF-D) is effective in restoring functional collecting lymphatic vessels in mice (Lahteenvuo et al., 2011; Tammela et al., 2007). As CCBE1 combined with VEGF-C results in significantly increased lymphangiogenesis, co-treatment may constitute a considerably improved strategy for promoting lymphangiogenesis. Furthermore, CCBE1 might also be a potential target for inhibiting lymphangiogenesis. Since it has been shown in pre-clinical tumor models, that inhibiting lymphatic vessel growth is sufficient to reduce lymph node and distant metastasis (Caunt et al., 2008; Kawada and Taketo, 2011; Laakkonen et al., 2007; Roberts et al., 2006).

In summary, our findings provide first insights into the possible mode-of-action of CCBE1is, and would suggest that CCBE1 acts as permissive factor for LEC migration and is of similar importance for lymphangiogenesis than VEGF-C.

MATERIAL AND METHODS

Mice

Mice were maintained at the Hubrecht Institute and Genentech, and experiments were performed according to national rules and regulations. LoxP mice were generated in which exons 4 and 5 of *Ccbe1* (comprising almost the entire Ca-EGF domain)

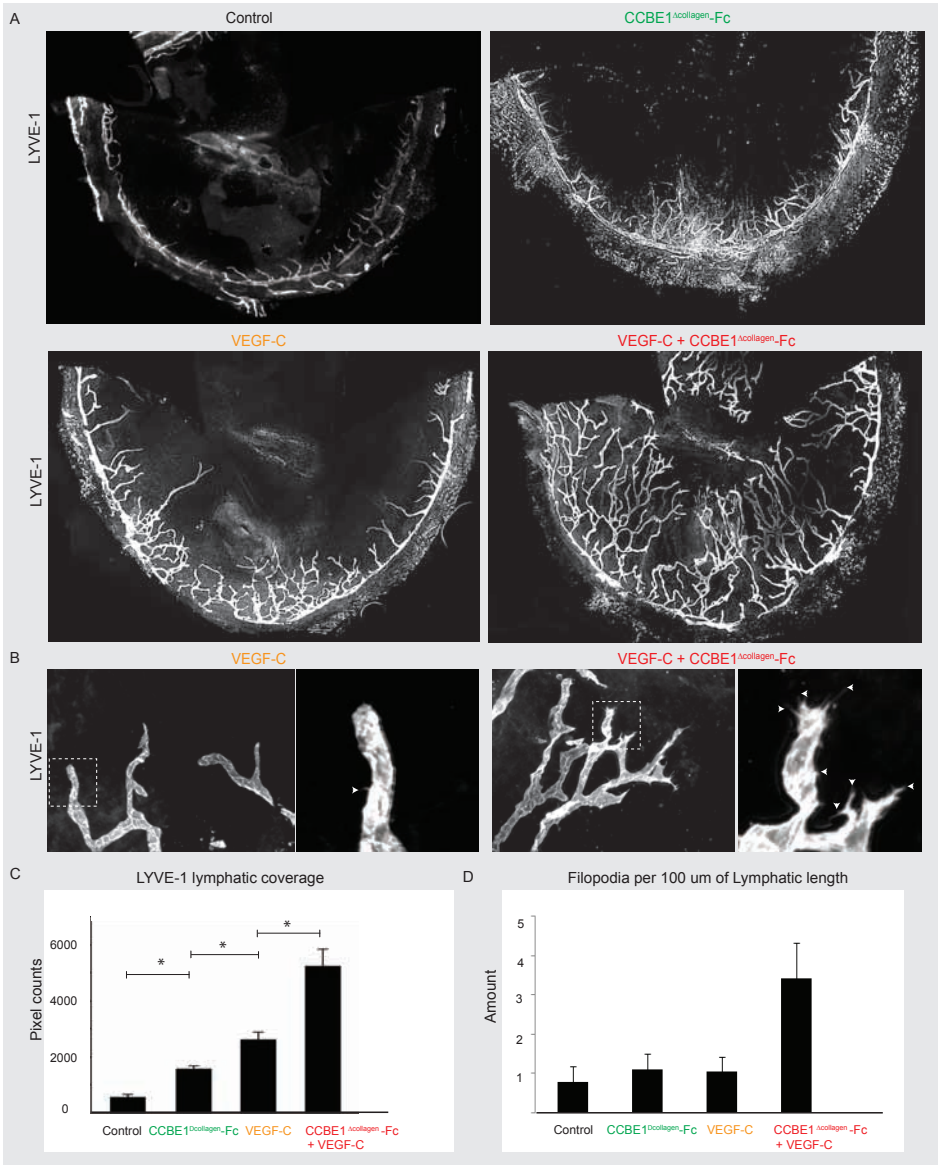

Figure 5: CCBE1$^{\Delta collagen}$-Fc modulates lymphangiogenesis. A, B. Representative images of LYVE-1 stained cornea, illustrating the effects of intracorneal placement of a control pellet and pellets of VEGF-C, CCBE1$^{\Delta collagen}$-Fc, and CCBE1$^{\Delta collagen}$-Fc + VEGF-C, respectively (A, upper right) A significant number of lymphatic endothelial cells have migrated towards a pellet of CCBE1$^{\Delta collagen}$-Fc. (A, lower left) VEGF-C elicits a stronger response of lymphatic endothelial sprouts. An increase in lymphangiogenic sprouting is seen when CCBE1$^{\Delta collagen}$-Fc and VEGF-C are combined (A, lower right). (B) High resolution images of filopodia formation in VEGF-C and VEGF-C + CCBE1$^{\Delta collagen}$-Fc treated corneas. Note the increased degree of filopodia formation in the combined treatment compared to VEGF-C alone (arrowheads). C. Quantification of LYVE-1^{+} pixel counts from the corneal micro-pocket assay shown in A. * p value < 0.05. D. Quantification of number of filopodia per 100um of lymphatic length shown in B.

were flanked by LoxP sites (*Ccbe1*$^{loxP/loxP}$). This allele was crossed to a general *Cre* recombinase strain (*PGK:Cre*), resulting in deletion of exons 4 and 5 and a predicted protein consisting of the N-terminal 90 amino acids. LacZ mice were generated by homologous recombination in embryonic SC with a *lacZ* cassette, which replaces the first and second coding exons of *Ccbe1* (*Ccbe1*^{lacZ}) (Tang et al., 2010). Both alleles were bred into Bl6 background.

Detection of β-galactosidase activity

Freshly obtained samples were fixed in a 2% paraformaldehyde/0.2% glutaraldehyde/PBS solution at RT for 30min. Samples were then washed 2 times with rinse buffer (2 mM MgCl2/0.1% NP40/PBS) and stained for 24 h in a solution consisting of 1 mg/ml X-gal, 5 mM ferrothiocyanide, 5 mM ferrithiocyanide in rinse buffer. Paraffin embedded samples were sectioned at 4 to 6 μm.

Immunohistochemistry

Whole mount tissues were fixed in 4% PFA and blocked prior to antibody staining. 6-25 μm paraffin or cryo-sections were blocked with 0.1%BSA, 5% milk or 10% goat or donkey serum, depending on type of tissue. Antibodies were incubated for 1hr to 24hrs, depending on the Ab. Samples were stained with Alexa 488, 568, 647 conjugated secondary antibodies (1:500; Molecular Probes) for 1 hr. Images were captured using Zeiss Axiophoto Fluorescence microscope, Leica SPE or Leica SP5 confocal miscroscopes. Antibodies used are LYVE-1 (R&D Systems), VEGFR3 (R&D Systems), PECAM-1 (BD and Chemicon), Prox1 (Chemicon).

Proximity ligation assay

Proximity ligation assay was performed as previously described (Soderberg et al., 2006) using the Duolink II Detection Kit (Olink Bioscience) and following primary antibodies: goat anti-VEGFR3 (R&D Systems), mouse anti-phospho-tyrosine 4G10 Platinum (Millipore) and rabbit anti-Lyve1 (Abcam).

Purification of the CCBE1$^{\Delta collagen}$-Fc fusion protein

Recombinant human CCBE1$^{\Delta Collagen}$ (NP_597716), residues (1-191) was expressed as a fusion with the Fc domain of human IgG1 from a transient transfection vector under control of the CMV promoter in Chinese Hamster Ovary (CHO) cells. Expression media containing human CCBE1-Fc was conditioned with 1mM Sodium Azide, and 0.5mM Phenylmethylsulfonyl Fluoride (pH pH 7.0), loaded over Protein A resin (ProSep-A, Millipore), washed and subsequently eluted with 50mM Sodium Citrate, pH 3.0. pH adjusted fractions (pH 5.0) were further purified by cation exchange chromatography (SP Sepharose FF, GE Healthcare) and eluted by a 0-1.0M NaCl elution gradient. Proteins were concentrated (10000 MWCO Amicron Ultra, Millipore) and dialyzed (10000 MWCO dialysis cassette, Pierce) into 25mM Tris/0.15M NaCl/2mM $CaCl_2$,

pH7.5. Final protein samples were sterile filtered and characterized by SDS-PAGE, O.D. 280, endotoxin assay, laser light scattering, and mass spectrometry.

Mouse corneal micro-pocket assay

Adult (8-10 weeks old) CD1 mice were anesthetized using isoflurane. A 1-2 mm initial cut was made on the cornea surface near the center using a #15 surgical blade. A micropocket directed toward the limbus is created by blunt dissection using a modified microspatula (ASSI 80017). Growth factor pellets are inserted (inert hydron, 0.2mmx0.2mm) to the base of the pocket, approximately 1 mm from the limbus. Growth factors used were: CCBE1$^{\Delta collagen}$-Fc (Genentech) at 500ng/pellet, VEGF-C (Genentech) at 250ng/pellet, or in combination. After 10 days, corneas were harvested and processed as described (Caunt et al., 2008).

ECM binding studies

HUVECs were plated onto 4-well chamber slides and allowed to reach confluence and deposit ECM for 3 days. Medium was removed and wells washed with 0,5% sodium deoxycholate to remove cells. Cells were briefly fixed in 4% PFA and then blocked in 1% BSA in PBS for 1 hr CCBE1$^{\Delta collagen}$-Fc or anti-Nrp2 antibodies at 10ug/ml incubated overnight. Wells were washed and IHC was performed. Collagen I, IV, V, fibronectin, laminin, vitronectin or BSA was coated on ELISA plates overnight. Plates were washed with PBS and blocked in 1% BSA for 3 hours. Increasing concentrations of CCBE1$^{\Delta collagen}$-Fc, anti-Nrp2 antibodies, or Alk1-Fc were incubated overnight. Plates were washed in 0.05% tween-20/PBS and then incubated for 1 hour with anti-human-HRP 1:5000 in 1%BSA/0.05%tween-20/PBS. Plates were washed in PBS 0.05% tween-20 and then incubated in TMB in the dark for 20 minutes. 2N sulfuric acid was added to stop the reaction and plates read at 450nm (n=3 per condition).

Contributions

F. L. B carried out the mutant analysis and wrote the manuscript. M. C. performed the in vitro characterization of the recombinant protein. L. P-P and E.L. designed and carried out the proximity ligation assay. J. P-M, J. Korving and J. H. v. E generated the loxP knock out allele. J. Kowalski performed the corneal micropocket assay. T. K. and J. K. helped with the mutant analysis. T.K and A. van I isolated mutant zebrafish *ccbe1* alleles, which were analyzed by A. van I.

ACKNOWLEDGEMENTS

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Judy Mak and Weilan Ye provided key support. Weilan Ye and A. McInnes provided useful comments.

SUPPLEMENTAL FIGURES

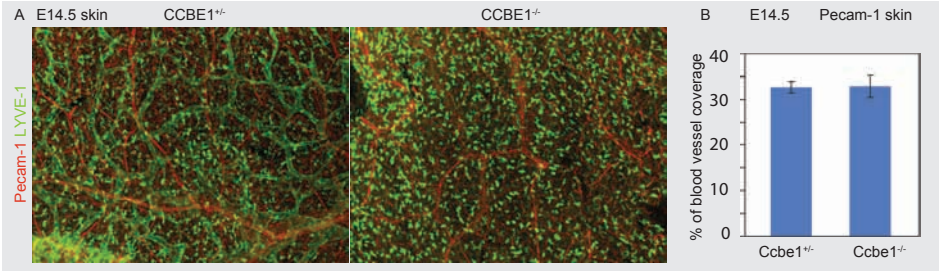


Figure S1: Blood vessels are not affected in Ccbe1 deficient mice. A. LYVE-1 (green) /PECAM-1 (red) staining of E14.5 *Ccbe1*$^{-/-}$ skin which lack lymphatic vessels but retain LYVE-1^{+} macrophages. B. Quantification of PECAM-1^{+} vessel coverage of the skin at 10 different regions.

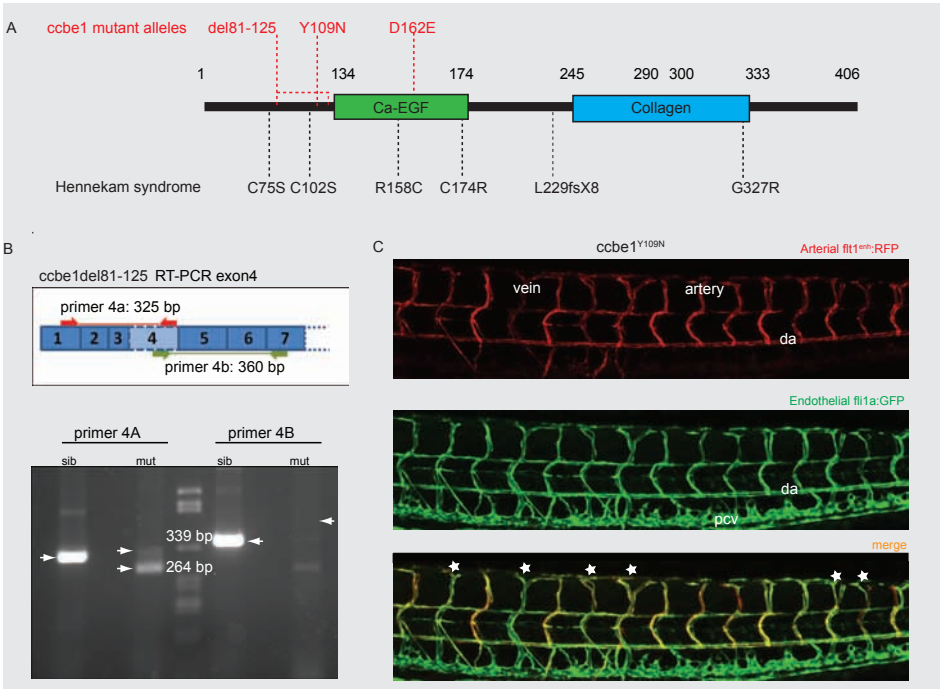
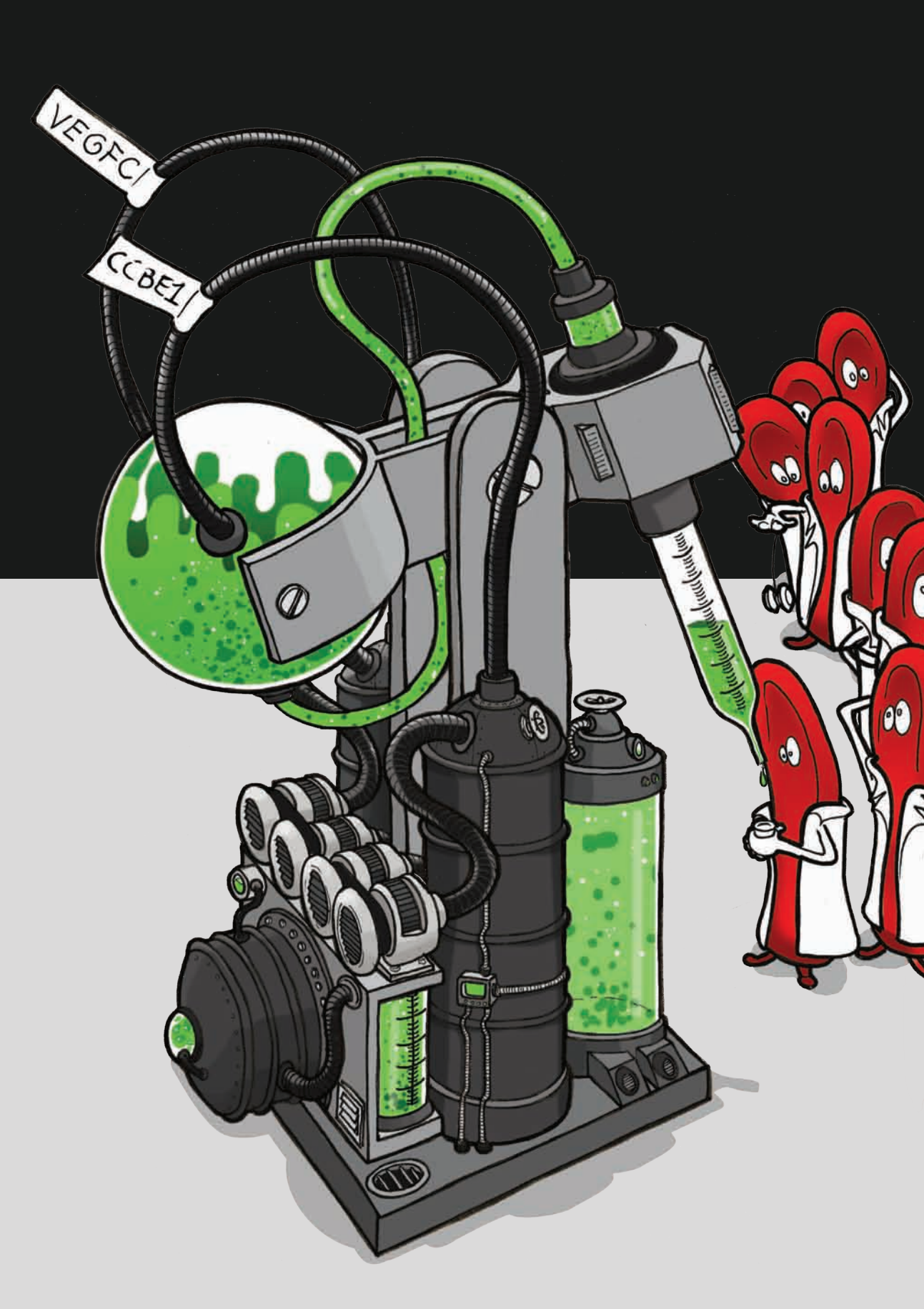


Figure S2: Overview of mutations in human Hennekam Syndrome and zebrafish *ccbe1* mutants. A. Schematic presentation indicating the currently known human and zebrafish *ccbe1* alleles. B. Characterization of novel zebrafish mutant *ccbe1*$^{del81\text{-}125}$ showing in frame deletion of the Ca-EGF domain and altered *ccbe1* transcript levels validated by RT-PCR. (C) Zebrafish trunk vasculature at 5dpf visualized by double transgenic labeling of arteries and veins. Note the existence of venous intersegmental vessels (stars) in *ccbe1*^{Y109N}.


VEGFC
CCBE1

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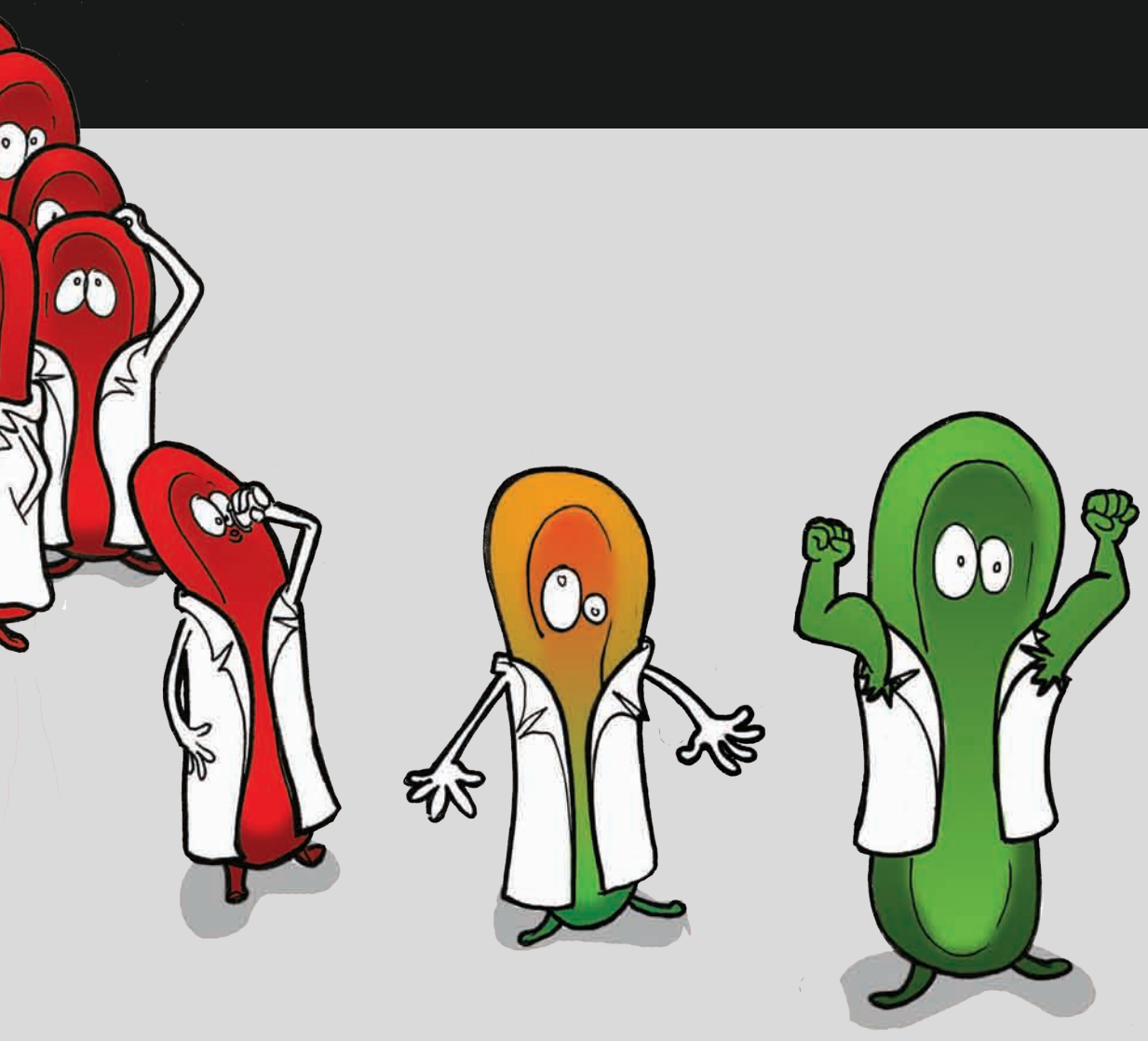
SUMMARIZING DISCUSSION



DISCUSSION

In this thesis we describe key aspects of the genetic regulation of zebrafish and mouse vascular networks. Particularly, we were interested in understanding the first steps of endothelial assembly into vascular tubes (vasculogenesis), sprouting of endothelial cells (angiogenesis) and establishment of the lymphatic vasculature (lymphangiogenesis).

Screening for novel regulators of vascular development

In chapter 2, we have performed a genome wide micro-array analysis for genes specifically expressed in *flk-1+ (Vegfr-2)* endothelial cells during crucial timepoints of murine vascular development. Since we used mouse embryos at E10.5 and E11.5 for our screen setup, the amount of vasculogenenis genes identified was rather limited. This is likely since vasculogenesis s very intensive before these timepoints (Drake and Fleming, 2000). Therefore, using earlier timepoints (E6.0-E9.5) would possibly reveal more genes that specifically regulate vasculogenesis, instead of angiogenesis or lymphangiogenesis genes.

In this setup, we further validated these genes in zebrafish by an *in situ* hybridization screen and subsequent morpholino knock-down and mutant analysis. The expression screen resulted in the identification of a number of genes specifically expressed in vascular endothelium, e.g. *thsd1, plvap, lgmn, tnfaip8l1* and *erg*. Of these genes, only depletion of *tnfaip8l1* resulted in a vascular defect. Furthermore, knockdown of *thsd1* resulted in specific hemorrhaging in the head region of zebrafish in a dose dependant manner. However, vascular patterning as such developed completely normal. Also in mice, *Thsd1* is also expressed in the vasculature as well as in haematopoietic stem cells, but a possible vascular function has not been described (Takayanagi et al., 2006).

In addition, we also found genes with expression restricted to venous endothelium. We identified *lyve-1* as a marker for zebrafish venous endothelium and, in addition, we found that *stabilin-1* and *stabilin-2* exhibit a very similar expression pattern. Since *lyve-1* is an established marker for lymphatic vessels in mice (Gale et al., 2007) and since in mice venous endothelium is also a precursor tissue for lymphatic endothelium (Srinivasan et al., 2007), we further explored the expression and function of *stabilins*. In zebrafish, the exact origin of lymphatic endothelium was still poorly understood. Now, with the transgenic reporter line of *stabilin-1:YFP in vivo,* and time-lapse imaging, we could further characterize the contribution of venous endothelium towards lymphatic endothelium in zebrafish lymphangiogenesis.

Stabilins in zebrafish development

In chapter 3, we developed a *stabilin-1:YFP* transgenic line and followed expression of the gene during embryonic development. With high resolution *in vivo* imaging, we found that *stabilin-1:YFP* labels initially the whole endothelium, subsequently becomes restricted to venous endothelium, and ultimately also labels all major lymphatic structures in adult zebrafish. To characterize the role of *stabilin-1* and *stabilin-2* during

zebrafish vascular development we also generated stable mutants of *stabilin-1* and *stabilin-2*. Both *stabilin-1* and *stabilin-2* mutants were viable with normal vascular patterning and development. However, *stabilin-1/stabilin-2* double mutant zebrafish are embryonic lethal between 6 dpf and 14dpf. A detailed characterization of the double mutants revealed that the initial haematopoiesis is normal, but double mutants become progressively anemic after 5dpf. In zebrafish, haematopoiesis occurs in two waves, namely a primitive (24-48 hpf) and a definitive wave (4-5dpf). In the definitive wave, the location of the adult hematopoietic stem cells shifts towards the kidney, where the adult and lifelong haematopoiesis is established. Strikingly, even though many hematopoietic mutants have been identified in zebrafish, only a few mutants have been described that become progressively anemic around 5dpf, pointing towards a defect in erythrocyte cell expansion or erythrocyte stability (de Jong and Zon, 2005). A detailed analysis of haematopoietic stem cell and erythrocyte maturation markers should reveal at what embryonic or larval stage haematopoiesis is altered in *stabilin* double mutants.

Recently, it was shown in mice that *Stabilins* are directly involved in clearance of waste products and toxic macromolecules by the endothelium (Schledzewski et al., 2011). Usually, most of this clearance is solely done by *Stabilin-1* and *Stabilin-2,* which are expressed on liver sinusoidal endothelial cells (LSEC). Genetic evidence from mice lacking *Stabilin-1* or *Stabilin-2* revealed that single mutants are phenotypically normal, which resembles the observation with our mutant zebrafish. However, mice lacking both *Stabilin-1* and *Stabilin-2* have a decreased lifespan and harbor severe fibrosis of kidney structures, whereas the liver showed only mild fibrosis (Schledzewski et al., 2011). The embryonic lethality of the zebrafish double mutants and the decreased lifespan in the double knock-out mice suggest an important role of stabilins. Both animal models seem to indicate that at least one *stabilin* allele is required for proper tissue homeostasis. Taken together, we demonstrate here for the first time a potential role for *stabilins* in the late initiation of haematopoiesis and erythropoiesis in zebrafish. However, future research is required to understand the exact role of *stabilin* genes in the onset of anemia and definitive haematopoiesis.

The development of the lymphatic vasculature

In chapter 4, we provide the first in vivo demonstration of LECs to be dependent on arterial ECs for pathfinding. With the generation of novel transgenic lines expressing selective markers for specific vascular structures it was possible to study the relationship of angiogenic endothelial cells with lymphatic endothelium. The distribution of arteries and veins in the trunk of the zebrafish can now be visualized through the transgenic expression of arterial specific *flt1* (*flt1enh:RFP*). Combining the *flt1* transgene with specific lymphatic transgenic lines, we can visualize the distribution of arteries, veins and lymphatics in a single developing zebrafish embryo, enabling us to characterize angiogenic and lymphatic zebrafish mutants in extraordinary detail.

For instance, by introducing the angiogenesis mutant *Hu5088 (kdrl)* into such a transgenic background, we were able to show that in this mutant, the arterial angiogenic network was not properly developed. Also, only a partial thoracic duct was established. This indicated a possible role (direct or indirect) for *kdrl* in the onset of lymphatic development. However, *kdrl* is not expressed in LECs and furthermore, this interaction does not depend on *kdrl*, as in those few cases where arterial instersegmental vessels were observed in mutants, LECs could still migrate along arteries. This pointed at a role of arteries rather than a role of *kdrl*, and a prediction of this hypothesis would be that by deleting arteries by other means one might obtain an equally effective block of LEC migration in the trunk. Indeed, suppressing the arterial *Notch* pathway results in a shift from arterial to venous fate, resulting in a loss of arteries and a subsequent block in migration of LECs (Geudens et al., 2010). These findings are consistent with a model where arteries are required for proper LEC guidance and with the situation in *kdrl* mutants.

In mice, the guidance of LECs is known to require the *Vegf-c/Vegfr-3* signaling axis. In *Vegf-c* null mice, LECs are initially formed, but the migration of LECs cells is altered, indicating a function for *Vegf-c* in directing migration of LECs. Therefore, in zebrafish, the possible contact-dependent migration of LECs on arterial ECs provides a new paradigm for LEC pathfinding. Several possibilities could be explored to obtain more insight into the mechanism regulating the migration of LECs along arteries. The secretion of guidance factors by arterial ECs which can attract LECs, such as *Vegf-c*, is one possibility. The other possibility is that LECs and arteries share a preference for the same molecular environment or the same guidance cues. Furthermore, arterial ECs might deposit extracellular matrix components that provide crucial substrate components for LECs. Further studies will have to unravel which molecules are central to the interaction between these two types of endothelial cells.

Ccbe1 in zebrafish and mammalian lymphatic development

Other than the ability to follow individual cells *in vivo* due to the transparency of the embryo, zebrafish offer the possibility to perform forward genetic screens with the aim to identify gene functions based on their functional requirement for a particular biological function. In chapter 5, we have performed such screens and isolated the mutant *full of fluid (fof)*. Mapping and sequencing of *fof* resulted in the identification of a point mutation in the gene collagen and calcium binding EGF domains 1 *(ccbe1)*. We used the *stabilin-1:YFP* and arterial *flt1* transgenic line to characterize mutants and morphants of *ccbe1*. Zebrafish *ccbe1* mutants have aberrant venous sprouting and as a result, the lymphatics are not established, suggesting a crucial role for veins in the onset of lymphatic development.

There are a few differences between zebrafish and mouse lymphangiogenesis. In mice, the lymphatics are formed after the establishment of a functional venous system. In zebrafish, venous sprouts and lymphatic precursors emerge from venous endothelium at the same time. Half of the sprouts from the posterior cardinal vein will contribute

to the lymphatic system, whereas the other half will remodel arterial intersegmental vessels into venous intersegmental vessels. In addition, in mice, the expression of the master regulator *Prox-1* in LECs separates lymphatic endothelium from blood vascular endothelium. Also in zebrafish, evidence is presented for a possible role of *prox1* as lymphatic markers, but convincing evidence is currently lacking (Del Giacco et al., 2010; Yaniv et al., 2006).

Remarkably, shortly after the identification of *ccbe1* in zebrafish a genetic linkage was found between *ccbe1* and lymphedema-lymphangiectasia–mental retardation, also known as Hennekam syndrome (MIM 235510) (Alders et al., 2009). This syndrome is characterized by disturbances in lymphangiogenesis and disruption of the drainage of interstitial fluids into the cardiovascular system, which eventually results in lymphedema. Also, unusual facial characteristics and mental retardation is observed (Forzano et al., 2002; Gabrielli et al., 1991; Van Balkom et al., 2002), probably due to facial lymphedema *in utero*. In addition, still births have been reported, suggesting a lethal embryonic phenotype and hence the low incidence rate of this syndrome (Connell et al., 2010). Taken together, Ccbe1 is required for zebrafish lymphangiogenesis and is linked to the Hennekam syndrome in patients. To further explore the mammalian function and mechanism of action of Ccbe1, we used two independent knock-out strategies for *Ccbe1* in mice.

In chapter 6 we made use of the *Ccbe1LacZ* allele, in which a *LacZ* cassette was recombined in the first coding exon of Ccbe1, thereby disrupting the translational start site of *Ccbe1*. In these mice we could follow expression of *LacZ* during murine embryonic development. Although the expression of *LacZ* was very weak in many parts of the embryo, we observed expression around vascular structures, in certain brain regions and in the mesothelium of the heart. Using a different approach, we introduced LoxP sites before exon 4 and after exon 5 of Ccbe1 *(Ccbe1LoxP)* and deleted this region with a general *Cre* recombinase (*pgk:Cre*). Strikingly, when we analyzed homozygous mutant mice of the *LoxP* strain, mutant mice were born without signs of edema formation. However, the mutants died straight after birth, probably due to suffocation. A detailed analysis of the blood and lymphatic vasculature revealed that the overall patterning of the blood vasculature was normal, but we could not detect lymphatics in skin, diaphragm and mesenteries, suggesting that all lymphatics are absent. When we bred the mice onto a C57/Bl6 background, Ccbe1 mutants were embryonic lethal due to severe accumulation of edema at E16.5, suggesting a dramatic failure in the drainage function of lymphatics. Indeed, these mutants also lacked the complete lymphatic vasculature, but still retained a normally patterned blood vasculature. Apparently, the genetic background of mice is very important to acquire the edema and should be taken into account when generating mutant mice with a putative lymphatic function. Indeed, the *Sox18* null mice only displayed edema when bred to a specific genetic background (Francois et al., 2008; Hosking et al., 2009), again indicating differences in regulation of lymphatic development in different genetic strains.

When analyzing the *Ccbe1* mutants in detail, we could show that blood endothelial cells (BECs) are still able to differentiate into LECs, as indicated by the appearance of

Prox1 expression cells. However, the migration and proliferation of LECs is altered and the specified LECs are lost over time, thus not capable of establishing the primary lymph sacs. The migration of LECs is dependent on the *Vegfr-3/Vegf-c* signaling pathway. In *Ccbe1* mutants we could not detect differences in *Vegfr-3* phosphorylation, one of the receptors for *Vegf-c*, indicating that *Vegf-c* is still able to signal via *Vegfr-3*, suggesting that guidance of LECs still occurs.

Ccbe1 contains a large collagen and calcium EGF domain and this might possibly suggest binding to several collagens, possibly components of the extracellular matrix (ECM). Strikingly, we were able to show that human recombinant CCBE1 protein, which lacks the collagen domain (CCBE1$^{\Delta collagen}$-Fc) is capable of binding to a subset of ECM proteins, such as Collagen and Vitronectin. These data are consistent with a model where CCBE1 does not physically interact with VEGF-C and VEGFR3, but rather changes the composition of the ECM.

A clue for the function of Ccbe1 might come from the observation that CCBE1$^{\Delta collagen}$-Fc binds specifically to Vitronectin. Vitronectin is a key ECM component implicated in complex signaling events that can lead to spreading, budding and migration of cells through the ECM (Smith and Marshall, 2010). One of the crucial components of this tissue remodeling is the urokinase-type plasminogen activator receptor (uPAR), the expression of which is up-regulated during tissue remodeling and in certain types of cancer. For remodeling, a specific protease is required to degrade the ECM before migration and budding of cells can take place. One of these proteases is the urokinase uPA. The receptor for uPA needs co-receptors for proper functioning and it has been shown that several integrins are essential for the signaling of uPAR. In addition, the ECM component Vitronectin is also a ligand for uPAR (reviewed in (Smith and Marshall, 2010). Since CCBE1$^{\Delta collagen}$-Fc can bind to Vitronectin, it is therefore possible that Ccbe1 is a direct or indirect activator of uPAR signaling via its binding to the ECM. Ccbe1 can possibly disrupt the ECM by uPAR signaling and provides a route for LECs to migrate through the ECM.

In addition to the collagen domain of CCBE1, the protein contains conserved EGF binding sites. CCBE1$^{\Delta collagen}$-Fc elicits a weak lymphatic response in the corneal micropocket assay, but this response is increased further in combination with VEGF-C. In general, EGF domains have been implicated to bind to the ECM, thereby acting as a crucial growth factor for sprouting and migration. For example, EGFL7 is expressed in endothelial cells, but it functions by binding to the ECM components collagen IV and is required for sprouting angiogenesis (Nichol et al., 2010; Parker et al., 2004; Schmidt et al., 2007). This result strengthens the ability that CCBE1$^{\Delta collagen}$-Fc can modulate the lymphatic endothelial cell behavior and is important in lymphangiogenesis.

Clinical benefits

Our findings that Ccbe1 plays a role in lymphangiogenesis may be of clinical relevance. For example, in cancer, tumor cells can spread towards lymph nodes (Figure 1A). To avoid this spreading, surgery of lymph nodes and radiotherapy is required. This

treatment damages the lymphatic system and is the leading cause of lymphedema (Figure 1B). The only treatment for lymphedema at present is the usage of garments, liposuction and physiotherapy (Tammela and Alitalo, 2010). Therefore, restoration of lymphatic function would be of great clinical benefit. The possibility of improving

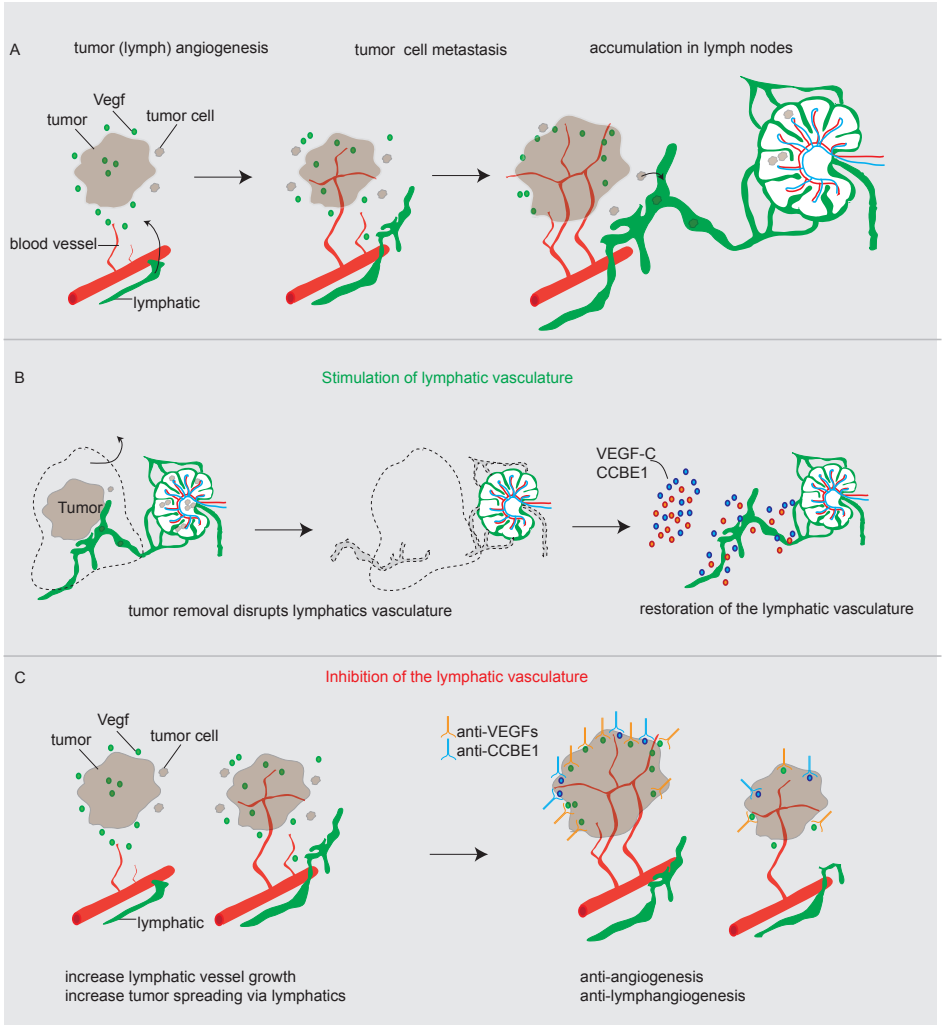


Figure 1: Therapeutic possibilities. A. In cancer, tumors can increase the production of vascular endothelial growth factors and thereby stimulate the growth of blood and lymphatic vessels. Then, tumor cells can spread easily towards lymphatics vessels and lymph nodes. B. Current therapy is based on surgery of lymph nodes and radiotherapy. This treatment damages the lymphatic and blood vasculature. Restoration of lymphatics can be done by enhancing the VEGF-C driven lymphatic response with CCBE1. C. Production of vascular endothelial growth factors can stimulate the growth of lymphatic vessels and spreading towards lymph nodes. CCBE1 can be a potential target for inhibiting tumor induced lymphangiogenesis. Anti-CCBE1, in combination with existing treatments, might be an attractive treatment.

lymphatic function has already been explored in mice. With gene transfer of the potent lymphangiogenic factor *Vegf-c* in mice, it has been shown that it is possible to reduce edema and re-stimulate lymphatic capillaries (Alitalo et al., 2005). When combining VEGF-C with another potent lymphangiogenic factor VEGF-D, it is also possible to further develop lymphatic capillaries into collecting lymphatics (Tammela et al., 2007). Our finding that CCBE1$^{\Delta collagen}$-Fc can further enhance the VEGF-C driven lymphatic response in the corneal micropocket assay might suggest that Ccbe1 could provide an additional tool to stimulate lymphatic vessel growth. Ultimately, combining VEGF-C with CCBE1 would possibly stimulate a lymphangiogenic response and may contribute to restoration of lymphatic vessels and the connection to lymph nodes and improve clinical outcome of lymphatic associated diseases (Figure 1B).

On the other hand, Ccbe1 might also be a potential target for inhibiting lymphangiogenesis. In pre-clinical tumor models, it has already been shown that it is possible to inhibit lymphatic vessel growth by ablation of lymphatic vessels and anti-lymphatic gene therapy, such as anti-VEGF-C regimes (Tammela et al., 2011) (Caunt et al., 2008; Kawada and Taketo, 2011; Laakkonen et al., 2007; Roberts et al., 2006). These methods and treatments were able to reduce lymph node and distant metastasis of cancer cells. Therefore, anti-CCBE1 treatment may be an attractive treatment as well. Improving the anti-lymphangiogenic response by multiple treatments might eventually lead to improved clinical outcome of certain cancer patients (Figure 1C).

The identification of CCBE1 as a protein that on the one hand may have clinical benefits to restore lymphangiogenesis and on the other hand as a target for inhibition of lymphangiogenesis may have clinical relevance, but it is only the first step towards the development of a new treatment. Much research is still required to understand the underlying mechanisms of action of CCBE1 and to validate possible clinical potential. This research will benefit from the generation of further mouse and zebrafish models with selective mutations in Ccbe1 and from the identification of more mutations in CCBE1 in Hennekam syndrome patients.

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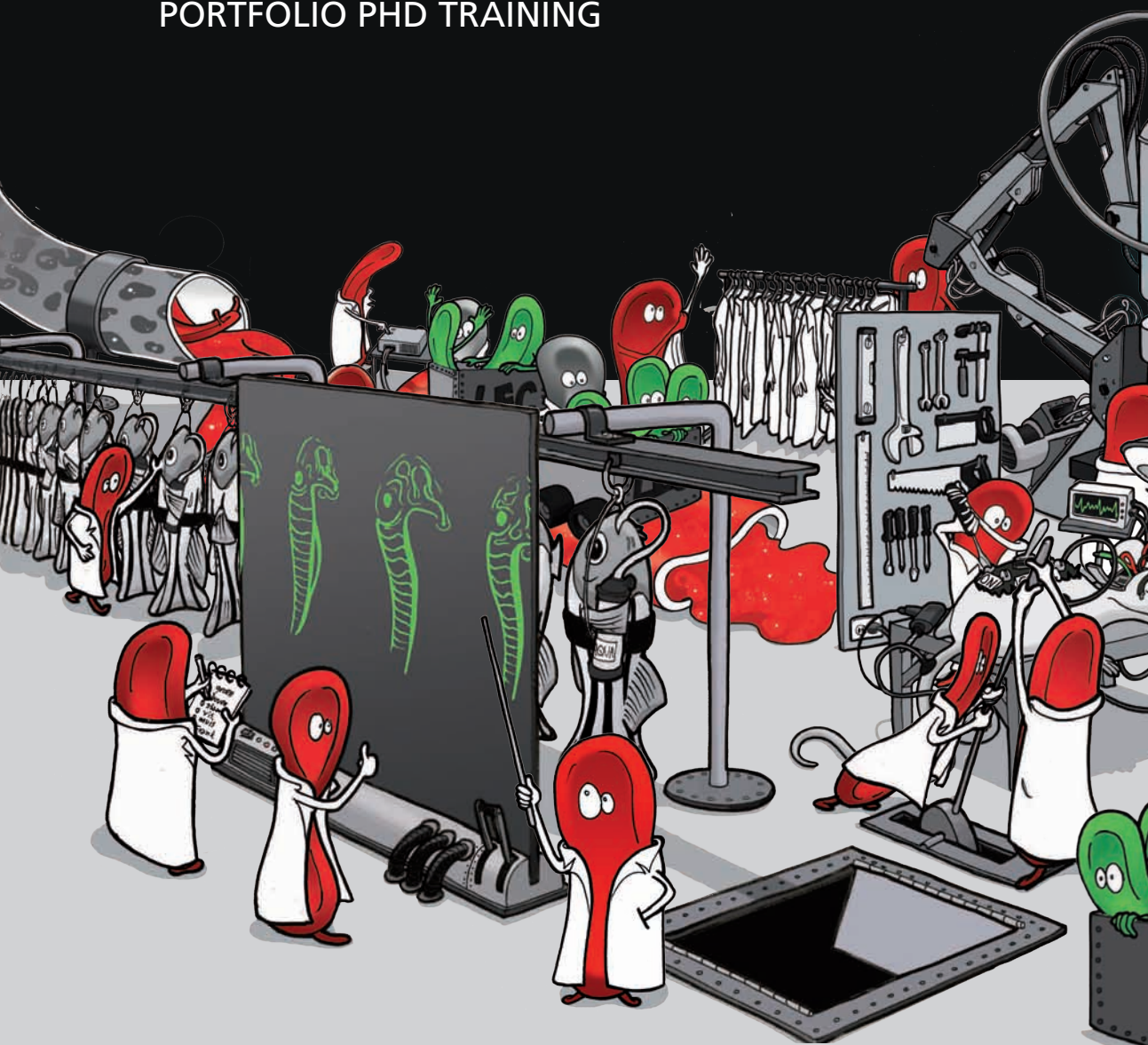

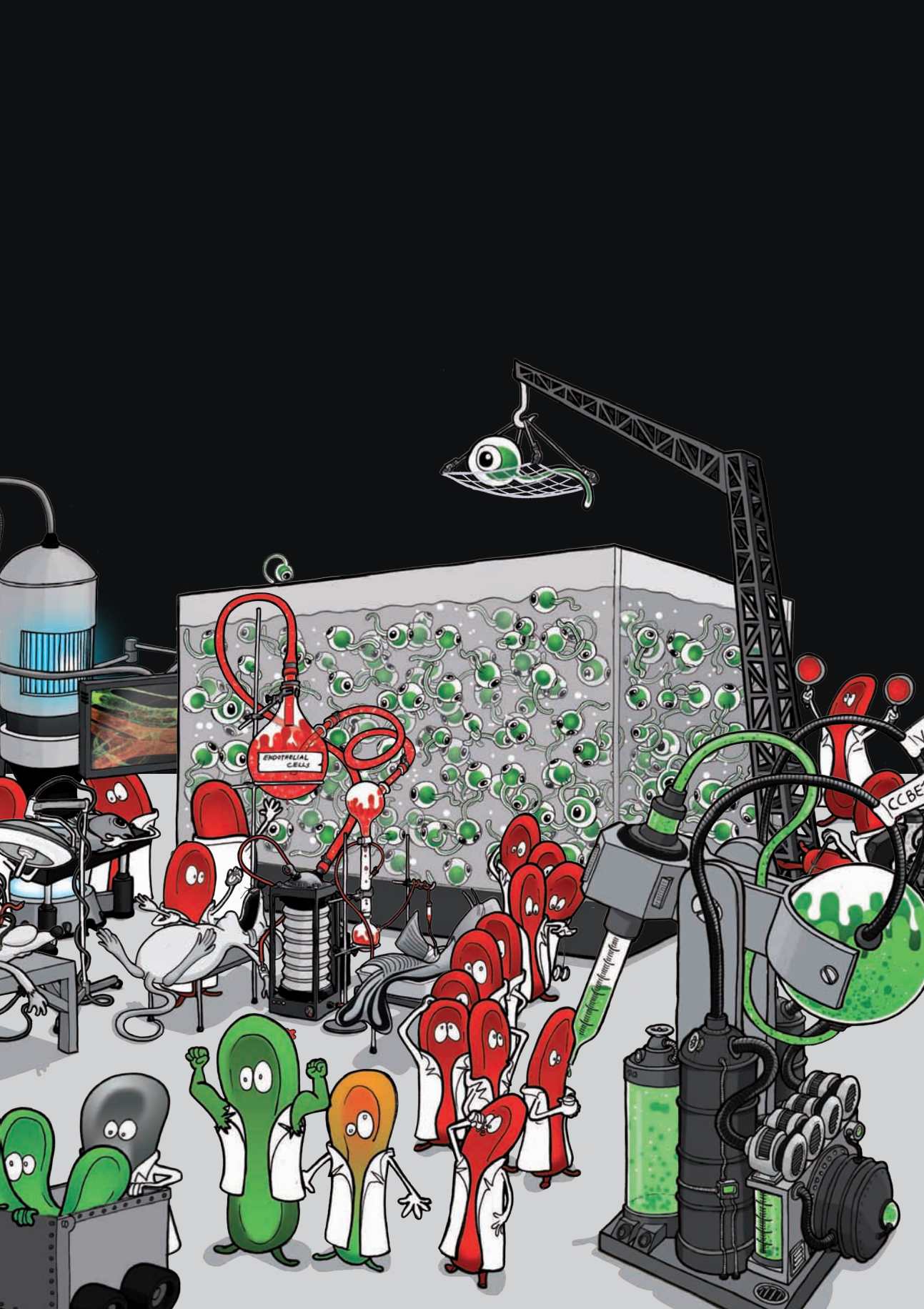
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SAMENVATTING

Waarom bestuderen we de ontwikkeling van bloedvaten en lymfevaten?

Een goed functionerend netwerk van bloed- en lymfevaten is essentieel voor de ontwikkeling van een organisme. Een beter begrip van het daadwerkelijk ontstaan van bloed- en lymfevaten is van cruciaal belang. Door slecht functionerende bloedvaten kun je een hartaanval of een hersenbloeding krijgen. Maar, als we kunnen achterhalen hoe bloedvaten precies gevormd worden, kunnen we deze kennis gebruiken om eventuele schade te voorkomen of mogelijk te repareren.

Normaal gesproken worden vaten alleen aangemaakt tijdens de embryonale ontwikkeling. Bloed-en lymfevatvorming vinden echter ook plaats bij de ontwikkeling van kanker. Bij kanker gaat een (kanker) cel ongecontroleerd snel delen waardoor een klompje ''slechte" cellen ontstaat (een tumor). Om verder te groeien hebben deze cellen zuurstof nodig. Om daarin te voorzien zorgen tumoren ervoor dat ze doorbloed raken. De tumor maakt daartoe bepaalde groeifactoren die normaliter alleen bestemd zijn voor het aanmaken van bloedvaten tijdens de embryonale ontwikkeling. In sommige gevallen willen we dus ook de vorming van bloed- en lymfevaten remmen om zo tumorvorming een halt toe te roepen of de ontwikkeling van kanker tegen te gaan.

Het hart en de bloedvaten zorgen voornamelijk voor het transporteren van bloed en voedingstoffen door het hele lichaam. Door de bloeddruk in de bloedvaten wordt een gedeelte van de vloeistof buiten de vaten in het weefsel geperst. De lymfevaten werken als een soort drainage systeem dat er voor zorgt dat de balans weer wordt hersteld. Bloedvaten kunnen op twee manieren ontstaan tijdens de embryonale ontwikkeling. Ze ontstaan vanuit enkele losse cellen die heel nauwkeurig samenkomen tot een buisvormige structuur (*vasculogenese*) en ze worden gevormd vanuit een al bestaand bloedvat (*angiogenese)*. Als een netwerk van bloedvaten is aangelegd, ontstaan daaruit vervolgens ook de lymfevaten en die ontwikkeling van lymfevaten wordt daarom *lymf-angiogenese* genoemd.

Uiteindelijk willen we precies weten welke stukje DNA (genen) belangrijk is voor de aanmaak van nieuwe bloed- en lymfevaten. Voor snel genetisch wetenschappelijk onderzoek gebruiken wij de zebravis, het bekende zwart-wit gestreepte visje dat in veel aquaria te zien is. Hoe het bloed- en lymfevatenstelsel van een zebravis ontstaat, is namelijk vrijwel identiek aan dat van een mens. Dit komt omdat er een sterke overeenkomst is tussen het DNA van de zebravis en dat van zoogdieren. In het bijzonder, omdat de gehele embryonale ontwikkeling van de zebravis plaatsvindt buiten het lichaam en het embryo volledig transparant is. Dit maakt het mogelijk om de ontwikkeling van bloedvaten 'live' te zien gebeuren en tot in het fijnste detail te kunnen bestuderen. Binnen 24 uur is een volledig netwerk van bloedvaten aangemaakt en het hart begint dan ook al te kloppen.

Hoe kunnen we achterhalen welke genen er nu precies belangrijk zijn voor het aanmaken van bloed- en lymfevaten. In hoofdstuk 2 gebruiken we een aantal methoden om in de zoektocht van het totale aantal genen (25.000) naar ongeveer 10

kandidaat-genen toe te werken. Om die 10 genen te testen op hun betrokkenheid bij bloedvatontwikkeling, hebben we deze stuk voor stuk uitgeschakeld. Zodoende konden we een aantal genen vinden dat betrokken is bij het proces van bloedvatvorming. Een voorbeeld hiervan is het gen *Tnfaip8*. Bij uitschakeling van dit gen zien we dat de bloedvaten minder worden gevormd en wij concluderen daarom dat het *Tnfaip8* gen betrokken is bij bloedvatvorming.

Een ander voorbeeld zijn de genen *Stabilin-1* en *Stabilin-2*. Deze twee genen hebben we bekeken omdat ze heel specificiek aan staan tijdens de ontwikkeling van bloedvaten. In hoofdstuk 3 beschrijven we voor het eerst een functionele rol voor *Stabilin-1* en *Stabilin-2* in de zebravis. We hebben gebruik gemaakt van een techniek om *Stabilin-1* of *Stabilin-2* voor altijd uit te schakelen. Er gebeurt echter niets met de bloedvaten als *Stabilin-1* of *Stabilin 2* los van elkaar uitgeschakeld worden. Maar, het tegelijkertijd uitschakelen van zowel *Stabilin-1* als *Stabilin-2* heeft een dramatisch effect; de bloeddoorstroming in het embryo neemt af en het aantal bloedcellen dat zuurstof kan vervoeren neemt ook af. Hierdoor kan het embryo niet overleven. Bij dit onderzoek waren we dus oorspronkelijk op zoek naar genen die betrokken waren bij angiogenese, het blijkt echter dat *Stabilin-1* en *Stabilin-2* betrokken zijn bij de ontwikkeling van bloedcellen in de zebravis.

Naast het gericht uitschakelen van genen, hebben we ook heel nauwkeurig bekeken hoe nu eigenlijk het netwerk van bloedvaten in de zebravis onstaat. Met behulp van geavanceerde microscopie kunnen we heel specifiek de ontwikkelende lymfevaten bekijken. In hoofdstuk 4 hebben we eerst een gedetailleerd overzicht gemaakt van alle bloed- en lymfevaten in de zebravis. Daarbij hebben we een trucje gebruikt om bloedvaten 'aan te kleuren'. We hebben alle bloedvaten rood gemaakt en alle lymfevaten groen. Zo kunnen we de verschillende typen vaten van elkaar onderscheiden en in één oogopslag zowel de bloedvaten als de lymfevaten visualiseren. We kwamen tot de ontdekking dat de ontwikkeling van een lymfevat afhankelijk is van de aanwezigheid van een bloedvat. Het lymfevat navigeert als het ware op het bloedvat en gebruikt het als houvast om te kunnen groeien.

Na het nauwkeurig in kaart brengen van het lymfevatennetwerk konden we onderzoeken welke genen er betrokken zijn bij de aanmaak van lymfevaten in de zebravis. Wij wilden dit onderzoeken omdat lymfevaten essentieel zijn voor een organisme. Het is van belang om nieuwe lymfevaten aan te maken en weefsel weer gezond te maken na een beschadiging (na een operatie) of bij een afwijking van de lymfevaten. Bij mensen met een afwijking in het lymfevatenstelsel kan er lymfe-oedeem (vochtuittreding) ontstaan, wat leidt tot opzwelling van de ledematen. Tot nu toe is hier nog geen behandeling voor. Een extreem voorbeeld hiervan is elefantiasis, ook wel 'olifantenziekte' genoemd, omdat de opgezwollen benen van de patiënten worden vergeleken met de poten van een olifant.

Van alle 25.000 genen die er zijn in de mens, is er maar een handjevol dat de aanmaak van lymfevaten reguleert. In hoofdstuk 5 hebben we onderzoek gedaan naar een zebravis die een mutatie heeft in een gen dat essentieel is voor de vorming van

lymfevaten. We hebben de mutatie kunnen terugvinden in het gen CCBE1, een gen met een tot nu toe onbekende functie.

Na het publiceren van de rol van CCBE1 bij de ontwikkeling van lymfevaten in de zebravis door ons lab heeft een andere onderzoeksgroep een aantal patiënten gevonden dat mutaties in CCBE1 heeft. Deze patienten hebben voornamelijk vochtuittreding door niet goed gevormde lymfevaten. Om dit verder te onderzoeken, hebben we in hoofdstuk 6 een muismodel gebruikt, waarin we CCBE1 hebben uitgeschakeld. Deze muizen hebben ook geen lymfevaten en zijn daarom niet levensvatbaar. Hiermee tonen we aan dat CCBE1 ook in zoogdieren een essentiële rol speelt bij de aanmaak van de lymfevaten.

Klinische relevantie

Tot slot bespreek ik de klinische relevantie van ons onderzoek. Het vinden van nieuwe genen, die essentieel zijn bij de aanmaak van bloed- en lymfevaten, kan nieuwe aangrijpingspunten geven om heel gericht medicijnen te ontwikkelen. Voornamelijk het gen CCBE1 is een goede kandidaat. CCBE1 is een groeifactor die, in combinatie met een ander lymfevat als stimulerende factor, heel waardevol kan zijn. De functie van CCBE1 zou ook geblokkeerd kunnen worden om de aanmaak van lymfevaten af te remmen.

Wij zijn van mening dat in beide gevallen de kennis van dit onderzoek gebruikt kan worden voor het ontwikkelen van geneesmiddelen voor het bestrijden van vaatziekten en kanker.

DANKWOORD

Promoveren kun je vergelijken met een goed opgebouwde DJ set. Het begint rustig en je weet niet wat er gaat komen. Als je er eenmaal helemaal in op gaat, kun je er lange tijd van genieten. Je laat je meerdere keren meevoeren naar een climax, maar je krijgt geen moment rust. De DJ eindigt vaak met een paar rustige nummers totdat de beats rustig uitdoven. Het ultieme dance gevoel.... En zo voelt het nu ook. Het boekje is af. Heerlijk.

Promoveren doe je voor jezelf, maar je kunt het echt niet helemaal alleen. Iedereen die mij heeft geholpen, op welke manier dan ook, heel erg bedankt! Ik heb met veel plezier gewerkt op het Hubrecht onder begeleiding van Stefan Schulte-Merker en aan het Erasmus MC onder begeleiding van Eric Duckers en Prof. Duncker.

Eric, het was geweldig om bij je groep te horen. Het klikte vanaf het begin al goed en het is erg mooi om te zien hoe je elk resultaat direct aan een ziektebeeld of patient kunt koppelen. Bedankt voor al je steun en vertrouwen.

Stefan, I have learned so much from you during my PhD and really appreciate everything you have done for me. For both of us the switch from fish to mice during my PhD was a big risk. Finally, after some hectic moments it resulted in the first mouse publication from your lab. You are a great person to work with.

Jeroen, jouw snelheid van denken en je relaxte manier van werken is een briljante combinatie. Je had altijd tijd voor me en je wist (bijna) alles. Snel weer eens een keertje fietsen in de bergen!

Ben, you are a great guy and person to work with! Next MOvember will be in San Francisco!

Robert, je hebt me heel veel geholpen bij het opzetten van mijn eerste projecten. Je had altijd de mooiste proeven en het onderzoek ging je goed af. Altijd zeer kritisch en oprecht; klasse! Binnenkort maar weer naar een meeting met 'Hot-tub' in de sneeuw?

Kirsten, je vrolijkheid en gezelligheid waren zeer nodig en jij was veruit de verstandigste in ons kantoor. Ik regel bitterballen op mijn feestje.

Ellen, de 'oudste' van het lab (sorry). Bedankt voor alle tips, trucs, uitleg en gezellige KGB'tjes. Whistler was geweldig!

Ive, je bent zeer belangrijk voor het lab. Altijd leuk en gezellig met veel lol en leuke gesprekken tijdens onze koffie 'dates'.

Josi, altijd positief en gouden handjes. Aan het muizen project kon ik zonder jou niet eens beginnen. Bedankt!

Terhi, with your great help and expertise, I could not wish for a better person to talk about the mouse work.

Matthias, Stefan en **Cornelia**, bedankt voor al jullie hulp en inzet tijdens de stages.

Bas, sorry voor al mijn dance-muziek. Met baard mag je niet op mijn feest komen.

Guy, 'Mister Biochemistry' en de 'Messi van het Hubrecht'. Het was bijzonder om onder jouw gezag te mogen spelen. Volgend jaar kampioen worden met 7 tegen 7!

Past and present lab members

Leonie (favorite post-doc!?) **Merlijn, Andreas**, **Dörthe, Bettina, Erik, Akihiro, Chrissy, Jo, Shijie, Eirinn, Evisa, Dorien** and **Alexander**. Thanks for great times in and outside the lab. Good luck with all your careers!

De Rotterdam-groep: **Caroline, Remco, Dennie, Esther en Lau**. Allemaal bedankt voor tips, hulp en gezelligheid.

Alle animal care takers, super bedankt voor het meehelpen aan het onderzoek. **Jeroen K.** (de dropman) en **Harry,** bedankt voor al het snij- hak- en denkwerk.

Verder voor iedereen die dit dankwoord leest en denkt, waarom sta ik er niet in? Op vrijdag 21 oktober zal ik je persoonlijk bedanken. Af te halen aan de bar.

Ontspanning & vrije tijd

Het grote nadeel van bezig zijn met de wetenschap is dat er steeds een mannetje in je hoofd zit, die af en toe eens roept: ''he, Frank!, zou je dit niet eens doen", en ''heb je dat wel gedaan?" Constant bezig zijn met je werk: soms is het fijn, soms verschrikkelijk. Hoe zorg je dan voor ontspanning? De slogan van Olympos zegt het al; ''als je fit bent, denk je beter!" Sporten dus!

Samen met o.a. **Jeroen**, **Hugo** 'ik zak twee keer door de squashbaan' en met **Lucas** 'met zweetbandje verlies ik niet'. Fanatiek squashen (=racket kapot slaan) en praten over proeven en resultaten. Vooral de laatste twee jaar van mijn promotie waren erg zwaar, mede omdat ik tot het uiterste moest gaan om niet te verliezen met squashen (en daar heb ik een hekel aan). Naast het squashen ook nog wielrennen met **Bart, Erik, Sjoerd** en **Arnout** (op de gele banaan), en met vele anderen tuffen over de 'Utregse' Heuvelrug. Heerlijk. Heren fietsers, bedankt voor de ontspanning (of inspanning).

Erik, mooi dat we het laatste half jaar precies hetzelfde schema hadden met submitten, re-submitten, paper accepted, boekje schrijven en promoveren.

Arnout, biertje drinken, bootje varen, dance-feestje, bedrijf beginnen... Dit gaat nog heel mooi worden.

Dan ook nog het twee-wekelijkse Hubrecht-voetbal-uurtje. **Heren voetballers**, allemaal bedankt! Nu is het voetballen bij het Hubrecht van zo'n hoog niveau dat ik de laatste jaren bij Hercules Utrecht extra moest trainen om dit niveau te halen..... Zaterdag is en blijft de mooiste dag van de week. Heerlijk om met vrienden een beetje achter een bal aan te rennen in een weiland. Heren van **Hercules 4 (Bolwerk United)**: super bedankt voor alle mooie zaterdagen en derde helften! In 2015 ben ik er weer bij met o.a. Plan Frank II. Ok, nog eentje dan...

Naast het sporten is het heel belangrijk om vaak af te spreken met vrienden en familie om ook eens wat te begrijpen van de niet-wetenschappelijke wereld. Altijd maar in het lab staan is ook niks.

Diederik, vanaf groep 6 zijn we al goede vrienden. Vele avondjes uit, festivals, hapjes eten, Begur, Bali. Allemaal erg mooie momenten in ons leven. Je hebt altijd zeer veel interesse in mijn werk, dat waardeer ik enorm. Bedankt!

Marco, Roland, Ivo, Kristel, Monique en **Bianca:** Legendarische weekendjes in de Ardennen en tijdens het WK in Zeeland. Jullie zijn echt leuk!

Dr. Geert en **Anna, JP, Dr. Hugo** en **Wouter**; mooi om samen te beginnen aan de studie en 12 jaar later allemaal te eindigen met Hora Est. Over een jaar kunnen we elkaar aanspreken met Doctor. Een mooi vooruitzicht!

Hugo, op het Hubrecht zochten we elkaar vaak op voor een wandelingetje. Vaak om even de dag door te nemen, een beetje klagen over de wetenschap, altijd fijn. Ik ben heel blij dat je doorgaat met wetenschap. Buiten het werk om, met jou en **Eveline** lekker skiën in Italië en Oostenrijk en af en toe een borreltje!

Wouter, de 3 maanden in San Francisco gaan nog steeds de boeken in als één van de mooiste en gaafste zomers in mijn leven. Gelukkig hebben we de foto's nog.... Bay to Breakers vergeet ik nooit meer: 'the chicken needs a beer'!

Hugo en **Wouter**, super dat jullie naast me willen staan tijdens de verdediging!

Familie

Gelukkig heb ik ook nog heel wat familie rondlopen aan wie ik erg gehecht ben, waaronder mijn oudere broers Herman en Wouter. Nadat **de vader** was begonnen met fietsen, ben ik ook maar eens gaan trappen in en rond SF. Het virus verspreidde zich makkelijk en mijn broers hadden het ook te pakken. Nou, mensen, ga een keer met de **familie Bos** fietsen en je maakt wat mee. Het is onmogelijk om een band-dikte voor te rijden met je fiets, want dat is het teken om gas te geven (is dat een demarrage?). Vaak eindigt dit in een moordend tempo (in de 'bergen' van NL) om vervolgens boven aan in een weiland gestrekt te gaan en aan het infuus te hangen, al wachtend en toekijkend hoe de vader langs fietst met de opmerking: 'hup doorfietsen jongens'. Zo gaat het ongeveer elke keer. Maar prachtig dat het is!

Herman, broer, beste vriend, fantastisch om met jou, je lieve vrouw **Floor** en jullie prachtige dochter Sophie eens lekker bij te kletsen en lekker gewoon doen. Fijn om jullie tot nu toe dicht bij mij te hebben. San Francisco is dan wel wat ver, maar daar vinden we wel wat op.

Sophie, als je dit over een paar jaar leest: oom Kak (Oom Frank) is nog steeds kek (gek). Hier zal niets aan veranderen!

Wouter, in jouw dankwoord voorspelde je dat mijn proefschrift in 2023 af zou zijn na de aanschaf van de PS3. Het is iets eerder geworden, excuses. Samen met **Marleen** heb je het goede leven te pakken. Het is altijd fijn om weer bij jullie te zijn. Het weekendje met wijnproeverij en Dance-Valley was een mooie afleiding tijdens het schrijven van mijn boekje. Bedankt!

Opa en Oma, bedankt voor het nalezen en verbeteren van mijn Nederlandse Samenvatting en natuurlijk voor alle gezellige avondjes in een van de restaurants in Utrecht. De volgende keer in San Francisco!

Een zoon van een professor zijn is niet altijd makkelijk.... dacht ik op het moment dat ik college van je kreeg. Een volle collegezaal, **mijn vader**, en iedereen weet dat hij je vader is. Nou, ik weet niet wie er zenuwachtiger was, jij of ik. Als jij het verprutst heb

ik geen vrienden meer. Na een minuut wist ik het: Dit kun jij als de beste! Wetenschap zit in je 'aderen'. Terugkijkend op alle mooie jaren tijdens studie en promoveren, waarin we echt alles konden bespreken. Samen de summer schools op Spetses organiseren was (en blijft) fantastisch. In 2015 ben ik er weer bij.

Mam, eerst kies je voor een man die in de wetenschap zit, vervolgens heb je ook nog een jongste zoon die hem achterna gaat. Sorry. Gelukkig hebben we het ook wel eens niet over die akelige wetenschap en kunnen we gewoon lekker bijkletsen over echt belangrijke dingen. Heerlijk om af en toe even naar Bunnik te fietsen voor een lekkere maaltijd, een geweldige espresso en veel gezellige avondjes met veel gespreksstof en wijn. Ik waardeer het enorm dat **jullie** altijd voor me klaar staan, altijd interesse hebben en precies weten wat er speelt. Super!

Yvonne, je bent echt geweldig. Samen gaan we een fantastische tijd hebben in San Francisco en heerlijk van elkaar genieten. Ik heb er zo veel zin in. Jij bent de beste!

Het feest kan beginnen,

Dr. Bossie is binnen

Traveling somewhere, could be anywhere
There is a coldness in the air, but I don't care
We drift deeper into the sound, life goes on
We drift deeper into the sound, feeling strong
So bring it on.
So bring it on…

Embrace me, surround me
As the rush comes,
As the rush comes,
As the rush comes.

LIST OF PUBLICATIONS

Ccbe1 is essential for mammalian lymphangiogenesis and enhances the effect of VEGF-C in vivo
Frank Lukas Bos, Maresa Caunt, Josi Peterson-Maduro, Lara Planas-Paz, Joe Kowalski, Terhi Karpanen, Andreas van Impel, Jeroen Korving, Johan H. van Es, Eckhard Lammert, Henricus J. Duckers & Stefan Schulte-Merker
Circulation Research, July 2011, in press

Arteries provide essential guidance cues for lymphatics endothelial cells in the zebrafish trunk
Frank Lukas Bos*, Jeroen Bussmann*, Akihiro Urasaki, Koichi Kawakami, Henricus J. Duckers and Stefan Schulte-Merker
Development, August 2010; 137(16):2653-7
* Authors contributed equally

ccbe1 is required for embryonic lymphangiogenesis and venous sprouting
Benjamin M. Hogan, Frank Lukas Bos*, Jeroen Bussmann*, Merlijn Witte*, Neil Chi, Henricus J. Duckers , Stefan Schulte-Merker
Nature Genetics, April 2009; 41(4):396-398
* Authors contributed equally

Molecular causes for BUBR1 dysfunction in the human cancer predisposition syndrome mosaic variegated aneuploidy
Saskia J.E. Suijkerbuijk, Maria H.J. van Osch, Frank Lukas Bos, Sandra Hanks, Nazneen Rahman, Geert J.P.L. Kops
Cancer Research, June 2010; 70(12):4891-900

Ets2 Determines the Inflammatory State of Endothelial Cells in Advanced Atherosclerotic Lesions
Caroline Cheng, Dennie Tempel, Wijnand K. Den Dekker, Remco Haasdijk, Ihsan Chrifi, Frank Lukas Bos, Kim Wagtmans, Esther H. van de Kamp, Lau Blonden, Erik A.L. Biessen, Frans Moll, Gerard Pasterkamp, Patrick W. Serruys, Stefan Schulte-Merker, Henricus J. Duckers
Circulation Research, June 2011; 109:382-395

CURRICULUM VITAE

Frank Lukas Bos is geboren op 28 maart 1982 in Leiden, Nederland. In het jaar 2000 behaalde hij zijn VWO diploma aan het Sint Gregorius College in Utrecht. Vervolgens studeerde hij Medische Biologie aan de Universiteit Utrecht. In 2004 werd de Bachelor-fase met success afgerond. Aansluitend begon hij aan de master Cancer Genomics and Developmental Biology die in 2006 ook met succes werd afgerond. Tijdens deze master liep hij zijn eerste onderzoeksstage van 11 maanden in het Hubrecht Instituut in het lab van Prof. Dr. Hans Clevers, onder begeleiding van Dr. Madelon Maurice deed hij onderzoek naar de Wnt-signalerings route die kan leiden tot darmkanker. Daaropvolgend liep hij 7 maanden stage bij de onderzoeksgroep van Prof. Dr. Rene Medema. Onder begeleiding van Dr. Geert Kops onderzocht hij de rol van genen die belangrijk zijn in het verdelen van chromosomen tijdens de celdeling. Daaropvolgend liep hij 3 maanden stage bij Dr. Frank McCormick aan de Universiteit van San Francisico, waar hij werkte aan het vroeg detecteren van kanker. In november 2006 startte hij als onderzoeker in opleiding aan het Erasmus Medisch Centrum te Rotterdam in de onderzoeksgroep van Dr. Eric Duckers. Gedurende zijn gehele onderzoeksperiode was hij echter werkzaam aan het Hubrecht Instituut, onder begeleiding van Prof. Dr. Schulte-Merker. Tijdens zijn promotie was Frank ook 3 maanden werkzaam als gast-onderzoeker in het lab van Dr. Anil Bagri & Dr. Weilan Ye bij Genentech, te San Francisco. De resultaten van het promotie onderzoek zijn in dit proefschrift beschreven.

PORTFOLIO PHD TRAINING

Frank Lukas Bos,
Erasmus MC Cardiology & Hubrecht Institute
Research School: CGDB (Utrecht) and COEUR (Rotterdam)
Promotor: Prof. Dr. Dirk Jan Duncker and Prof. Dr. Stefan Schulte-Merker
Supervisor and co-promotor: Dr. H.J. Duckers

Type of Education	Year	ECTS
Seminars & Presentations	2006-2011	4
CGDB evenings	2006-2011	2
Courses CGDB		
Course Bioinformatics	2006	1.5
Signal Transduction Course	2007	1.5
Photoshop Imaging Course	2008	0.75
Illustrator Course	2009	0.75
Courses COEUR		
Molecular Biology in Cardiovascular Research	2009	1.5
Cardiovascular Medicine	2007	1.5
Meetings and Conferences		
Meeting ELSYS, Enschede, The Netherlands	2007	1
EMBO Signal Transduction Meeting, Spetses, Greece	2007	2
European Zebrafish Meeting, Amsterdam, The Netherlands	2007	1
Keystone Meeting Hypoxia and Angiogenesis, Vancouver, Canada	2008	1.5
Keystone Meeting Lymphangiogenesis and Cancer, Big Skye, USA	2009	1.5
NAVBO Meeting Angiogenesis and Lymphangiogenesis, Asilomar, USA	2010	1.5
PhD retreat and Masterclasses		
PhD retreat	2007-2010	3
Masterclass, Doorwerth, The Netherlands	2006-2009	3
Teaching and Supervision		
Supervision Master Student	2009	1.5
Supervision Master Student	2010	1.5
Total ECTS		**31**